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Tests with Fluorides, Especially Sodium Fluoride, as Anthelmintics for Swine

R. T. HABERMANN, D.V.M., F. D. ENZIE, D.V.M., and A. O. FOSTER, Sc.D.
Washington, D. C.

IN connection with the usual criteria by which all livestock anthelmintics are judged—safety, efficacy, ease of administration, and cost— a specific requisite of an anthelmintic suitable for swine is satisfactory action against the large roundworm, *Ascaris lumbricoides*. The importance attached to ascaricidal action is the natural result of the fact that such gastrointestinal parasites as stomach worms, nodular worms, and whipworms are of less economic consquence than the large roundworms. The thorn-headed worm, *Macracanthorhynchus hirudinaceus*, is of importance in some areas but it presents a special case not only on account of its limited distribution but also because no known anthelmintic has proved even partially useful for its removal.

Of the treatments that have been employed for controlling large roundworms of swine, oil of chenopodium (American wormseed oil) and phenothiazine, both of which were introduced as swine treatments by the United States Bureau of Animal Industry, are the only two that have shown sufficient, general usefulness to achieve wide recognition by hog raisers and veterinarians. Although phenothiazine came into use but recently and oil of chenopodium some years ago, the critical literature on the former and the clinical experience with it, are adequate to permit reasonably accurate evaluations of both drugs and fair comparisons between them (Enzie, Habermann, and Foster[3]). In brief, phenothiazine is less effective than oil of chenopodium in removing large roundworms, and its efficacy against these parasites is more variable. On the other hand, it has the advantages of easy administration and effective action against nodular worms. Probably the chief reason why some users prefer phenothiazine is that it can be given to a herd of pigs, in the feed, thus replacing individual treatments; also, there is no need for fasting animals before treatment or for purging them with or following the medication. Moreover, both of these standard anthelmintics for swine have given rise to instances of intoxication and death. In short, neither phenothiazine nor oil of chenopodium has, to a high degree, the attributes that are required in a general anthelmintic for swine.

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This work was carried out at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md.

HISTORICAL BACKGROUND

On account of the facts outlined above, the Zoölogical Division has continued to search for a more satisfactory anthelmintic for swine. Among the types of compounds tested were certain fluorine-containing substances, one of which—sodium fluoride—seemed especially promising.

Notwithstanding their known toxicity, it is natural that fluorides and fluorine compounds should be considered because the halogenated compounds, as a group, occupy a prominent place among anthelmintics. At least 250 compounds of this type have been explored, but the most useful and best known are certain chlorinated

hydrocarbons, such as carbon tetrachloride, tetrachlorethylene, hexachlorethane, and normal butyl chloride, all of which were originally introduced into the field of anthelmintics by the investigative work of this bureau. Moreover, some of the fluorides, as is well known, have proved especially useful as pest-control agents and insecticides, among them, sodium fluoride, sodium silicofluoride, and sodium aluminum fluoride (cryolite). In addition, recent work with the insecticidal aërosols has drawn attention to a group of mixed fluorine-chlorine hydrocarbons that are commonly called "freons" by chemists who are particularly interested in refrigerants. To the anthelminticist, the striking attributes of these substances are their relatively low toxicity to mammals in spite of their fluorine content and their close chemical similarity to chemicals of known anthelmintic action. Several years ago, Wright and Schaffer[10] of this bureau, tested trifluorotrichloroethane as an anthelmintic in dogs, finding it to be quite effective as an ascaricide, although somewhat irritating to the intestinal tract and bladder. Only a brief note on the testing of this "freon" was published.[5]

While it is probably a matter of common knowledge that fluorides have been used for years as pest-control agents, it is not as well know that the conception of their anthelmintic use is also relatively old. In reporting tests of the resistance of canine ascarid eggs, for example, Wigdor[9], in 1918, wrote: "Eggs kept in 1:2 solution of sodium fluoride, a salt which has recently been advocated as possessing valuable antiseptic properties, showed actively motile embryos at the end of three days." In extensive tests on the chemotherapy of oesophagostomiasis in sheep, Monnig[6,7], in 1933 and 1935, reported specifically on the anthelmintic action of sodium fluoride, sodium silicofluoride, calcium fluoride, and sodium aluminum fluoride (cryolite). All of these chemicals were administered internally to sheep in scientific experiments set up to determine certain aspects of the anthelmintic potentialities of miscellaneous substances. Shortly before the present studies were begun, our attention was called to a patent (Elmslie and Caldwell[2]) relating specifically to the use of fluorides in feed as anthelmintics for swine, although no data are given which can be construed as critical information on their efficacy in removing ascarids or other internal parasites from that animal. In fact, no scientific literature on this

TABLE 1—Data on Tests with Sodium Fluoride Administered to Swine for Three Days as a 1.0 Per Cent Mixture of the Feed

Animal	Wt. (Lb.)	Total Dose (Gm.)	Worms Removed		Worms Left	Efficacy (%)	Remarks
51	143.5	72.0	Ascarids	6	0	100	Diarrhea, enteritis; 12 whipworms eliminated in four days before treatment.
			Stomach worms	3	0	100	
			Nodular worms.	37	0	100	
			Whipworms ...	184	205	47	
52	19.0	14.5	Ascarids	2	0	100	Soft feces.
			Whipworms ...	0	3	0	
53	23.0	14.5	Ascarids	7	0	100	Soft feces.
			Whipworms ...	0	3	0	
54	27.0	19.8	Ascarids	0	2	0	Died on fourth day subsequent to start of treatment. Pneumonic lesions.
			Nodular worms.	0	23	0	
62	20.0	8.0	Stomach worms	2	0	100	Used previously as pig 60. (*see* table 2).
			Nodular worms.	0	87	0	
			Whipworms ...	0	5	0	
63	26.0	6.6	Ascarids	50	0	100	Used previously as pig 61. (*see* table 6).
			Stomach worms	10	124	7	
			Nodular worms.	3	7	30	
			Whipworms ...	5	5	50	
64	33.5	7.0	Stomach worms	6	4	60	Vomition.
			Nodular worms.	14	54	21	

subject seems to have been published. Recently, Cameron and Parnell[1] and Parnell[3] reported on results they had obtained with sodium fluoride, sodium silicofluoride, and certain other fluorides in the destruction of the infective larvae of bursate nematodes of horses and sheep.

The present report covers some preliminary investigations with the following fluorides: sodium fluoride, aluminum silicofluoride, barium fluoride, aluminum fluoride, sodium aluminum fluoride, and sodium silicofluoride. A brief account of some of the findings with sodium fluoride has already been published[4]. The studies are still in progress and, on that account, the findings should be interpreted as a progress account of tests conducted rather than as a recommendation for general anthelmintic use in swine. Nevertheless, the acute need for a suitable anthelmintic for these host animals raises the hope that other investigators will check the promise exhibited by certain of these compounds, especially sodium fluoride. The data are offered with full cognizance of the toxic potentiality of the fluorides, particularly sodium fluoride. It is also borne in mind that such successful anthelmintics as nicotine, oil of chenopodium, and carbon tetrachloride are also toxic, yet not so dangerous as to prohibit their use in accordance with established indications and methods.

MATERIALS AND METHODS

In these tests, 151 pigs were used in 185 trials. The pigs were of mixed breeds and ranged in age from approximately 3 months to 4 or 5 years. Most of them were less than 10 months old. Many were culls in poor condition; indeed, it was not uncommon to find 1 or more dead pigs in the lot containing the animals set aside for experimental use. Most of the pigs harbored natural infections of one or more of the intestinal helminths commonly found in swine in this locality; namely, large roundworms (*Ascaris lumbricoides*), stomach worms (*Ascarops strongylina*), nodular worms (*Oesophagostomum* spp.), and whipworms (*Trichuris suis*). In the preliminary critical tests, pigs were confined in individual, concrete-floored pens for one to nine days before treatment and the 24-hour accumulation of feces was screened daily to detect the natural elimination of parasites. The types of parasites in each animal were determined by fecal examination prior to treatment using the salt flotation method. One day before treatment, the pigs were usually given only about one-half of the regular feed, a prepared mash consisting chiefly of cracked yellow corn, bran, and oats; the substances tested were then given to the pigs in various percentages of the feed for periods of one to three days. This method of administration was chosen because of its simplicity. Usually, at the end of the test period, any medicated feed that remained was not removed but was mixed with various amounts of regular feed daily until all had been consumed. In critical tests, the feces were collected every twenty-four hours and examined for parasites. When the elimination of parasites ceased, the pigs were killed and their entire gastrointestinal tract examined for parasites and lesions. The other organs were examined macroscopically for evidences of pathological changes. In a few instances, groups of 20 or more pigs were treated with sodium fluoride primarily to ascertain the tolerance of comparatively large numbers of swine. In these cases, the feces were not screened as in the individual critical tests, but were examined daily for the presence of ascarids. At the end of the test period, the pigs were submitted to necropsy. A few tests were made in which mixtures of sodium fluoride and phenothiazine in various proportions were given to the pigs in the feed. At the end of the test period, most of these pigs also were examined *post mortem*.

In addition to the studies with swine, a limited number of tests were made with other animals: horses, cattle, sheep, goats, chickens, and dogs, primarily to ascertain the tolerance of these animals for sodium fluoride.

Representative tissue sections from several of the test animals were preserved for studies of their histopathology, in the event such examinations became indicated.

EXPERIMENTAL DATA

Experiment 1. One Per Cent Sodium Fluoride for Three Days (table 1).—In critical tests involving individual treatments, 7 pigs were given 1.0 per cent of the chemical in feed for three consecutive days. In general, the mixtures were readily eaten the first day, but became less palatable with each feeding. This treatment removed 65 (97%) of 67 ascarids, 189 (46%) of 410 whipworms, and 54 (24%) of 225 nodular worms from 5 pigs, and 21 (14%) of 149 stomach worms from 4 pigs. One animal, pig 54, died on the fourth day after the treatment was

started and the lesions at the necropsy suggested pneumonia as the primary cause of death. Another, pig 64, vomited on the first day, shortly after eating a portion of the medicated feed. The rest of the animals remained normal throughout the test period, although soft feces were passed temporarily by three pigs.

of the medicated mixture remained, until all was consumed. The treatment removed 100 per cent of 137 ascarids from 8 pigs; 552 (37%) of 1,476 nodular worms from 7 pigs; 2,492 (99%) of 2,513 stomach worms from 6 pigs; and 1 (4%) of 24 whipworms from 5 pigs. Pig 65 vomited shortly after eating a portion of the mixture. The

TABLE 2—Data on Tests with Sodium Fluoride Administered to Swine for One Day as a 2.0 Per Cent Mixture of the Feed

Animal	Wt. (Lb.)	Total Dose (Gm.)	Worms Removed		Worms Left	Efficacy (%)	Remarks
55	24.0	13.2	Ascarids	47	0	100	
			Nodular worms.	10	59	14	
58	32.0	13.2	Ascarids	13	0	100	
			Nodular worms.	5	4	55	
			Whipworms ...	0	1	0	
59	25.0	10.0	Ascarids	21	0	100	
			Stomach worms	1	0	100	
			Nodular worms.	311	733	30	
			Whipworms ...	0	14	0	
60	20.0	20.0	Ascarids	1	0*	100	Used subsequently as pig 62. (*see* table 1).
			Stomach worms.	1881	2*	99	
			Nodular worms.	33	87*	28	
			Whipworms ...	1	5*	17	
65	39.0	8.9	Ascarids	32	0	100	Slight vomition.
			Stomach worms	170	18	90	
			Nodular worms.	5	40	11	
			Whipworms ...	0	1	0	
66	28.0	10.0	Ascarids	8	0	100	
			Stomach worms	6	1	86	
67	59.0	18.0	Ascarids	10	0	100	
			Stomach worms	418	0	100	
			Nodular worms.	90	0	100	
70	27.0	10.0	Ascarids	5	0	100	Used previously as pig 68. (*see* table 6).
			Stomach worms	16	0	100	
			Nodular worms.	98	1	99	
			Whipworms ...	0	2	0	

*Calculated.

Experiment 2. Two Per Cent Sodium Fluoride for One day (table 2).—Although the ascaricidal action demonstrated in the above experiment was satisfactory, it seemed desirable for practical reasons to avoid the plurality of feedings if satisfactory anthelmintic action could be maintained otherwise. As a consequence, a number of experiments were conducted using various concentrations of sodium fluoride in the feed for one day. Eight pigs were given the drug individually as a 2.0 per cent mixture of the feed for one day. The mixture was eaten less readily than the 1.0 per cent mixture but, in most cases, it was consumed within two or three days. After the first day, regular feed was added daily to whatever portion

other animals remained normal throughout the test period.

Experiment 3. One and One-Half Per Cent Sodium Fluoride for One Day (table 3).—In individual, critical tests, 6 pigs were given 1.5 per cent of the chemical in the feed for one day. In most instances, the entire mixture was consumed the first day. The treatment removed 122 (99%) of the 123 ascarids from 4 pigs; 707 (96%) of 731 stomach worms from 5 pigs; and 1,146 (45%) of 2,491 nodular worms; and 8 (3%) of 240 whipworms from 6 pigs. Pigs 72 and 73 vomited shortly after consuming a portion of the mixture. The other pigs, however, were unaffected by the treatment.

Administered to a group of 4 pigs, at

the rate of 1.5 per cent, the medication removed 100 per cent of 17 ascarids and 13 stomach worms, 425 (35%) of 1,221 nodular worms, and 1 (2%) of 40 whipworms. Most of the mixture was consumed the first day, and there were no untoward reactions.

Treated as a group, 21 pigs were given 1.5 per cent sodium fluoride in feed, and the medication repeated after an interval of one week. The mixture was eaten quite readily in each instance. The animals passed 15 to 20 ascarids (precise number not ascertained) after the first test but only 1 worm was recovered when the treatment was repeated. No ascarids were found at the necropsy. Several of these pigs vomited before and after treatment, but their appetite was not affected.

Another group of 29 pigs was given 1.5 per cent sodium fluoride in feed, and the mixture was eaten quite readily. Although the feces were not screened, 48 ascarids were recovered after treatment and none was found *post mortem*. One pig exhibited symptoms of pneumonia on the third day after treatment. At the necropsy two days later, extensive consolidation of the lungs and adhesions between the pulmonary and parietal pleurae were found. Apparently, this condition was present before treatment. Several pigs vomited after eating a portion of the mixture, but most of the animals were unaffected, and in no instance was the appetite impaired.

Experiment 4. One Per Cent Sodium Fluoride For One Day (table 4).—Two pigs were given 1.0 per cent of the

TABLE 3—Data on Tests with Sodium Fluoride Administered to Swine for One Day as a 1.5 Per Cent Mixture of the Feed

Animal	Wt. (Lb.)	Total Dose (Gm.)	Worms Removed		Worms Left	Efficacy (%)	Remarks
69	55.0	15.0	Nodular worms.	42	0*	100	Used subsequently as pig 71. (*see* table 6).
			Whipworms ...	3	17*	15	
72	31.0	12.0	Ascarids	15	0	100	Vomition.
			Stomach worms	1	0	100	
			Nodular worms.	2	0	100	
			Whipworms ...	1	0	100	
73	25.0	12.0	Ascarids	53	0	100	Vomition; 63 nodular worms eliminated in ten days before treatment.
			Stomach worms	434	3	99	
			Nodular worms.	478	187	71	
			Whipworms ...	0	1	0	
80	26.0	7.5	Ascarids	52	0	100	Used previously as pig 75. (*see* table 6).
			Stomach worms	27	4	87	
			Nodular worms.	79	303	21	
			Whipworms ...	0	26	0	
81	49.5	12.0	Stomach worms	2	0	100	Used previously as pig 79. (*see* table 4).
			Nodular worms.	387	721	35	
			Whipworms ...	4	133	29	
82	30.5	9.0	Ascarids	2	1	67	
			Stomach worms	243	17	93	
			Nodular worms.	158	134	54	
			Whipworms ...	0	55	0	
86	58.0		Ascarids	17	0	100	3 immature ascarids eliminated in four days before treatment.
87	38.0	69.12	Stomach worms	13	0	100	
88	96.0		Nodular worms.	425	796	35	
89	37.0		Whipworms ...	1	39	2	
110-130	15-40	150.0	Ascarids	(a)	(b)	(c)	Retested one week later; several vomited before and after treatment.
		240.0	Ascarids	1	0	100	Several vomited after treatment.
131-159	20-45	270.0	Ascarids	48	0	100	Several vomited after treatment. One pig died of pneumonia.

(a) About 15-20 ascarids observed in feces.
(b) At least one ascarid.
(c) Probably compares favorably with other tests.
* Calculated.

drug individually in feed for one day. In one instance, the pig was fasted for 24 hours before treatment; both pigs ate the mixture readily. This treatment removed 204 (96%) of 211 ascarids from 1 pig; 230 (90%) of 255 stomach worms; 15 (1%) of 1,227 nodular worms; and none of 146 whipworms from both pigs. In 1 pig ascarids were not present. There were no untoward reactions.

A group of 19 pigs was given 1.0 per cent in feed for one day. Most of the mixture was eaten the first day. When a small amount of regular feed was added to the remainder, the pigs consumed it readily. The treatment removed 28 (97%) of 29 ascarids from the group. One runt died on the fourth day, and extensive lesions of pneumonia were found in both lungs; there was also a moderate hemorrhagic gastritis. Two pigs were in such poor condition that they were killed and examined *post mortem* on the fifth day. No specific reason for their condition was apparent other than general debilitation. Two pigs vomited after treatment but the others remained normal throughout the test period.

Five pigs were given this treatment and the mixture was eaten readily. The treatment removed all of 7 ascarids from the group. One pig vomited once but the others remained normal throughout the test period.

A group of 26 pigs was given the mixture for one day. These pigs were procured from a farm on which, according to the herdsman, losses had occured from erysipelas. Although none of these pigs was acutely ill, many had enlarged joints and areas of skin around them had sloughed away. Their appetites were apparently normal and the entire mixture of medicated feed was consumed the first day. The treatment removed all of 23 ascarids from the group. One or 2 pigs vomited several hours after the mixture was fed. One comparatively robust animal was found dead on the sixth day without having manifested any premonitory symptoms. At the necropsy, the lesions found were hemorrhagic gastroenteritis, nephritis, cirrhosis of the liver, and a marked congestion of all lymph glands. The rest of the animals remained normal throughout the test period.

TABLE 4—Data on Tests with Sodium Fluoride Administered to Swine for One Day as a 1.0 Per Cent Mixture of the Feed

Animal	Wt. (Lb.)	Total Dose (Gm.)	Worms		Efficacy (%)	Remarks
			Removed	Left		
79	49.0	8	Stomach worms 156 Nodular worms. 15 Whipworms ... 0	2* 1108* 137*	99 1 0	Fasted twenty-four hours before treatment. Used subsequently as pig 81. (*see* table 3).
83	25.5	5	Ascarids ..... 204 Stomach worms 74 Nodular worms. 0 Whipworms ... 0	7 23 104 9	96 76 0 0	
160-178	15-35	90	Ascarids 28	1	97	Two vomited after treatment. One pig died of pneumonia.
186-190	100-110	100	Ascarids 7	0	100	One pig vomited.
231-256	40-90	150	Ascarids 23	0	100	One pig died.

*Calculated.

Comment on Experiments 1 to 4

When sodium fluoride was given to pigs at the rates discussed previously, there were few significant untoward reactions. The chief effects attributable to the chemical were occasional vomition and softer feces, although these effects were transient. In all, 4 pigs out of the 124 used in 148 trials with sodium fluoride, died during the test periods but the lesions observed at the necropsies suggested the possibility that factors other than primary sodium fluoride poisoning were re-

sponsible for at least 3 of the deaths. One death may have been due chiefly to sodium fluoride toxicity. It was noted, moreover, that in the previous experiments (1 to 4) sodium fluoride was employed at rates of 2.0, 1.5, and 1.0 per cent of the feed, and that the results did not preclude the possibility that concentrations of less than 1.0 per cent may approach more nearly the optimum rate of administration. Moreover, pigs in better condition or of stronger type than those used in this study, might have sufficiently good appetites to eat enough of the chemical when it is given at less than 1.0 per cent of the feed mixture.

Experiment 5. Sodium Fluoride and Phenothiazine Mixtures (*table 5*).—In an attempt to remove a larger percentage of nodular worms, 17 pigs were given mixtures of sodium fluoride and phenothiazine in the feed. Sodium fluoride-phenothiazine ratios of 3:1, 2:1, 7:5, and 1:1 were given to swine at 1.2 to 2.0 per cent of the feed. In the aggregate, the treatment removed 100 per cent of 33 ascarids but only 559 (42%) of 1,309 nodular worms from the 12 helminth-infected pigs involved. In 2 pigs, 99 and 100, the efficacy was determined by fecal examination seven days after treatment. The feed mixtures containing both compounds were markedly less palatable

TABLE 5—Data on Tests with Various Mixtures of Sodium Fluoride and Phenothiazine Administered to Swine in the Feed

Animal	Wt. (Lb.)	Sodium Fluoride: Phenothiazine Mixtures with Respect to— Drug Ratio	Gm. Per Lb.	Per Cent in Feed	Total Dose (Gm.)	Parasites Removed		Left	Efficacy (%)	Remarks
92	33	3:1	0.4	2	60	Ascarids	2	0	100	About ⅔ of mixture consumed; 2 whipworms eliminated one day before treatment.
93	40					Stomach worms.	8	2	80	
94	75					Nodular worms.	1	19	5	
						Whipworms	6	0	100	
95	450	3:1	0.13	2	60					About ½ of mixture consumed.
96	24	2:1	0.45	1.5	52.5	Ascarids	23	0	100	About ⅔ of mixture consumed; 3 ascarids and 18 whipworms eliminated in four days before treatment.
97	36					Stomach worms.	25	1	96	
98	61					Nodular worms.	49	148	25	
						Whipworms	52	459	10	
99	150	2:1	0.196	1.5	60	Ascarids	6	?	..	Almost all of mixture consumed. Not autopsied, fecal exam. negative on seventh day after treatment.
100	150					Stomach worms.	14	?	..	
						Nodular worms.	2	?	..	
						Whipworms	3	?	..	
101	160	1:1	0.38	1.5	60	Ascarids	1	0	100	About ½ of mixture consumed; retested with sodium fluoride alone.
			0.21	1.0	35					NaF only. Almost all of mixture consumed; diarrhea. Not autopsied.
102	120	1:1	0.38	1.5	45	Stomach worms.	48	37	56	About ½ of mixture consumed; 1 nodular worm eliminated in five days before treatment.
						Nodular worms.	20	583	3	
103	75	7:5	0.4	1.2	30	Ascarids	1	0	100	About ⅔ of mixture consumed.
						Nodular worms.	1	0	100	
						Whipworms	0	7	0	
104	195	1:1	0.36	1.5	72	Stomach worms.	61	0	100	Molasses incorporated in mixture; about ⅔ of mixture consumed.
						Nodular worms.	488	0	100	
106	225	1:1	0.28	1.6	64					Molasses incorporated in mixture; about ½ of mixture consumed, about ⅓ remained on third day. Not autopsied.
107	20	1:1	0.4	1.7	24					Sour whey incorporated in mixture; most of mixture remained next day. Not autopsied.
108	20									
109	20									

than those in which only one compound was used. Moreover, those mixtures that were eaten readily were relatively ineffective against the nodular worm, and when the amount of phenothiazine was increased, the pigs refused to eat the mixtures.

Experiment 6. Tests with Miscellaneous Fluorides (table 6).—Because sodium fluoride has been considered a highly toxic compound, it seemed desirable to test other fluorides which might prove equally effective but less toxic. The specific compounds used in these tests were aluminum silicofluoride, barium fluoride, aluminum fluoride, sodium aluminum fluoride (cryolite), and sodium silicofluoride.

Aluminum silicofluoride was given to 1 pig as a 2.0 per cent mixture of the feed for one day. The entire mixture was eaten the first day. This treatment was wholly ineffective against 50 ascarids and 10 nodular worms and removed only 11 (8%) of 145 stomach worms and 2 (17%) of 12 whipworms. There were no untoward reactions.

Barium fluoride was given to 1 pig as a

TABLE 6—Data on Tests with Miscellaneous Fluorine Compounds Administered to Swine in the Feed

Animal	Wt. (Lb.)	Compound Tested	Treatment (Gm.)	Treatment (%)	Worms Removed		Worms Left	Efficacy (%)	Remarks
61	26.0	$Al_2(SiF_6)_3$	20.0	2.0	Ascarids	0	50*	0	Used subsequently as pig 63. (*see* table 1).
					Stomach worms.	11	134*	8	
					Nodular worms.	0	10*	0	
					Whipworms	2	10*	17	
68	27.0	BaF_2	15.0	3.0	Ascarids	19	5*	79	Vomition; used subsequently as pig 70. (*see* table 2).
					Stomach worms.	15	16*	48	
					Nodular worms.	1	99*	1	
					Whipworms	0	2*	0	
71	55.0	BaF_2	20.0[1]	2.0	Whipworms	0	17	0	Used previously as pig 69. (*see* table 3).
75	26.0	AlF_3	15.0	1.5	Ascarids	0	174*	0	Retested; 15 immature ascarids eliminated before treatment.
					Stomach worms.	1	9*	10	
					Nodular worms.	0	137*	0	
					Whipworms	0	7*	0	
			30.0	3.0	Ascarids	63	111	36	
					Stomach worms.	0	9	0	
					Nodular worms.	0	137	0	
					Whipworms	0	7	0	
75	26.0	AlF_3	7.5	1.5	Ascarids	0	52*	0	Retested.
					Stomach worms.	0	31*	0	
					Nodular worms.	0	382*	0	
					Whipworms	0	26*	0	
			15.0	3.0	Ascarids	0	52*	0	Used subsequently as pig 80. (*see* table 3).
					Stomach worms.	0	31*	0	
					Nodular worms.	0	382*	0	
					Whipworms	0	26*	0	
182	130.0	Na_3AlF_6	50.0	2.5	None	..	..	..	Retested.
			100.0	5.0	None	..	..	..	
183	100.0	Na_3AlF_6	100.0	5.0	None	..	..	..	
184	100.0	Na_3AlF_6	100.0	10.0	Ascarids	0	0*	..	Used subsequently as pig 185. Ascarid ova in fecal exam.
					Nodular worms.	0	63*	0	
					Whipworms	1	377*	0	
185	100.0	Na_2SiF_6	50.0	5.0	Ascarids	0	0*	..	Vomition; ascarid ova on fecal exam.
					Nodular worms.	0	63*	0	
					Whipworms	0	377*	0	
			20.0	2.0	Ascarids	0	0*	..	Vomition.
					Nodular worms.	0	63*	0	
					Whipworms	0	377*	0	
			10.0	1.0	Ascarids	0	0	..	Refused to eat mixture.
					Nodular worms.	0	63	0	
					Whipworms	0	377	0	
186	110	Na_2SiF_6	10.0	1.0	Ascarids	0	1	0	Vomition.
					Stomach worms.	4	0	100	
					Nodular worms.	9	1	90	

[1]Two days; *Calculated.

3.0 per cent mixture of the feed for one day and it was eaten quite readily. The treatment removed 19 (79%) of 24 ascarids; 15 (48%) of 31 stomach worms; 1 (1%) of 100 nodular worms; and none of 2 whipworms. Vomition occurred some time during the 24-hour period after treatment but there appeared to be no other reaction. A 2.0 per cent mixture for two consecutive days was ineffective against 17 whipworms in another pig. The mixtures were consumed readily and there were no untoward reactions.

Aluminum fluoride was given to 2 pigs as a 1.5 per cent mixture of the feed for one day. The mixtures were consumed promptly in each instance. The treatment was totally ineffective against 226 ascarids, 519 nodular worms, and 33 whipworms, and removed only 1 (2%) of 41 stomach worms from the 2 pigs. Five days later, this compound was given to these pigs as a 3.0 per cent mixture of the feed for one day. The mixtures were consumed promptly. This treatment removed 63 (36%) of 174 ascarids from 1 pig but none of 52 ascarids from the other. None of the 40 stomach worms, 519 nodular worms, and 33 whipworms was removed from the 2 pigs. There were no untoward reactions.

Sodium aluminum fluoride was given to 1 parasite-free pig as a 2.5 per cent mixture of the feed for one day, and, two days later, as a 5.0 per cent mixture. Both mixtures were consumed promptly without untoward reactions. Another parasite-free pig was given a 5.0 per cent mixture of the compound for one day with similar results. A 10.0 per cent mixture was equally well tolerated but removed only 1 of 378 whipworms and none of 63 nodular worms from 1 pig. One sheep weighing 60 lb. was given 30 Gm. of the compound in capsules. There was no anthelmintic effect or untoward reaction.

Sodium silicofluoride was given to 1 pig, at weekly intervals, in successive doses of 5.0, 2.0, and 1.0 per cent of the feed for one day. The treatments were wholly ineffective against 63 nodular worms and 377 whipworms in each instance. Ascarid eggs were found on fecal examination before treatment, but no ascarids were recovered after treatment and none was found at the necropsy. Profuse vomition occurred after the doses of 5.0 and 2.0 per cent, and the pig refused to eat the 1.0 per cent mixture. Another pig ate the 1.0 per cent mixture, but vomited shortly afterward. In this test the drug was ineffective against 1 mature ascarid, but removed all of 4 stomach worms and 9 of 10 nodular worms. One sheep weighing 40 lb. was given 10 Gm. of the compound in capsules. The animal died during the night. The necropsy revealed hemorrhages throughout the gastrointestinal tract.

Experiment 7. Toxicity Studies with Sodium Fluoride.—In view of the toxic nature of sodium fluoride, it seemed desirable to determine the symptomatology of acute fluorine poisoning, to ascertain the maximum tolerable dose of the compound for swine and for other animals, and to obtain data on any anthelmintic action the compound might have in these animals.

Swine.—The compound was given to 4 pigs as 4.0 or 5.0 per cent mixtures of the feed. One pig weighing 30 lb. was given 13.2 Gm. of sodium fluoride as a 4.0 per cent mixture of the feed. A little more than half of the medicated mixture was eaten by evening, when a small amount of regular feed was added to it. All of the mixture was eaten by the next morning, and the animal exhibited no unfavorable reactions. The pig died, however, on the sixth day, and the lesions observed at the necropsy suggested pneumonia as a highly contributory cause of death. The pig passed 5 mature ascarids spontaneously before treatment but none were recovered afterward. At the necropsy, 4 ascarids were found in the small intestine, a result that was unexpected in the light of earlier findings. Another pig weighing 130 lb. was given 78 Gm. of the compound at a similar concentration in the feed. After consuming the equivalent of approximately 20 Gm. of sodium fluoride, the pig vomited and thereafter refused to eat the mixture, although no other feed was given for five days. Other than vomition, this pig evinced no untoward reactions. After a fasting period of twenty-

four hours, a pig weighing 27 lb. was given 15 Gm. of the drug as a 5.0 per cent mixture of the feed. The pig ate the equivalent of 5 Gm. of sodium fluoride and then vomited several times. The next day, the pig was somewhat depressed, and a portion of the feces was quite soft. The animal had eaten the equivalent of another 5 Gm. of the drug, and, when a small amount of regular feed was added to the medicated mixture, the pig ate a small additional amount of it. In the afternoon, the animal was depressed and trembled constantly. The pig was found dead the following morning. At the necropsy, the most salient pathological lesion was a marked hemorrhagic gastroenteritis, and the liver and kidneys were markedly congested. It was interesting to note that 122 ascarids were either passed or found dead in the large intestine at the necropsy. One mature ascarid was found alive in the small intestine. A pig weighing 170 lb. was given 50 Gm. of sodium fluoride at a similar concentration of the feed. This animal had been given about one-half its regular feed the day before treatment, and virtually the entire mixture was eaten immediately. The pig did not vomit but became so depressed that it could not be forced to get on its feet. The pig died during the night, and at the necropsy the lesions found were identical with those observed in the preceding case.

Horses.—Eight tests were conducted with 7 horses to which single and multiple doses were given by capsule or in the feed. Horse 1 weighing 450 lb. was given 15 Gm. of sodium fluoride in capsules. The drug was given in 5-Gm. doses on alternate days. There were no untoward reactions, and 11 ascarids were recovered in the feces. Two months later, this animal was given 18 Gm. in capsules and 9 Gm. on each of two consecutive days. The only untoward reaction was diarrhea on the third and fourth days after treatment. Sixty ascarids and 58 oxyurids were recovered in the feces after treatment, and the ascarid-egg count was reduced from 2,600 to 0 eggs per Gm. Horse 2 weighing 1,300 lb. was given 10 to 20 Gm. in the feed daily for twelve days (except on the sixth and eleventh days), a total of 135 Gm. The only untoward reactions were slight inappetence and depression toward the end of the test period; many cylicostomes were found in the feces during the test. At the necropsy there was slight congestion of the stomach, and a few cylicostomes were found in the ventral colon. Horses 3 and 4 weighing 350 and 300 lb., respectively, were treated together. The drug, 36 Gm., was given in the feed, 18 Gm. the first day, 13.5 Gm. the fourth day, and 4.5 Gm. the fifth day. The only reactions to the treatment were slight inappetence and diarrhea after the first dose. The egg counts for horses 3 and 4 were reduced to 0 from 66 ascarid and 6 strongyle ova and 882 ascarid and 6 strongyle ova, respectively; 37, ascarids, 21 oxyurids, and 1 large strongyle were recovered in the feces after treatment. Horse 4 was submitted to necropsy and slight congestion of the small intestine was the only observable lesion; no ascarids were found. Horse 5 weighing 650 lb. was given 30 Gm. in the feed. Diarrhea and inappetence were observed on the second day after treatment, and the animal was destroyed *in extremis* on the third day. Post mortem examination revealed congestion of the stomach and submucosal hemorrhages in the small intestine and 6 dead ascarids and viable large and small strongyles in the large bowel. Horse 6 weighing 950 lb. was given 90 Gm. of sodium fluoride in the feed over a period of twelve days. The appetite was variable throughout the test period and the animal was somewhat depressed. After a lapse of five days, the horse was given a molasses-grain mixture in which 50 Gm. of the drug had been incorporated. The animal refused to eat the mixture although no other grain was offered for five days. The horse was submitted to necropsy, and the only lesions observed were small areas of congestion in the ventral colon and dark pigmented areas in the cortex of the kidneys. Horse 7 weighing 1,400 lb. was given 30 Gm. of sodium fluoride in grain; most of the mixture was eaten within twenty-four hours. There were no symptoms, and at the necropsy on the fourth day no lesions were found which could be attributed to the drug.

Cattle.—Two tests were conducted with 1 animal. Bull 243 weighing 400 lb. was given 40 Gm. of sodium fluoride in the feed, 15 Gm. the first day and 25 Gm. about two weeks later. In each instance, grain was refused the day after treatment but hay was eaten readily. Slight depression was observed after each treatment, but no other symptoms were noted. There was no significant anthelmintic action, and the necropsy revealed congestion of the small intestine.

Goats.—Seven goats were given sodium fluoride in capsules or in the feed. Two goats weighing 25 and 35 lb. were each given 5 Gm. of the drug in grain. The animals refused to eat the mixture so similar doses were given in capsules. One of the goats died during the night, and at the necropsy scattered hemorrhages were found throughout the gastrointestinal tract. Slight depression and soft feces were the only untoward reactions observed in the other animal, and no significant lesion could be detected at the necropsy held one week later. There was no apparent anthelmintic action in either case. Another goat of similar weight was given 5 Gm. in capsules. This animal died during the night, and at the necropsy the lungs were filled with a frothy material and hemorrhages were found in the reticulum and liver. One goat weighing 28 lb. was given 8 Gm. of the drug in the feed after a fast of thirty-six hours. The animal ate most of the mixture, and died during the night. At the necropsy hemorrhages were found throughout the internal organs. A similar dose given in capsules to a goat weighing 40 lb. was also fatal. The necropsy lesions were essentially the same as in the preceding animal. A goat weighing 150 lb. was given 15 Gm. in capsules, and marked depression and profuse salivation were observed within two hours. The animal died during the night and hemorrhages were found throughout the internal organs at the necropsy. Another goat weighing 135 lb. was given 10 Gm. of sodium fluoride in capsules. The animal was down and off feed the next day but gradually improved and was apparently normal on the seventh day. There was no apparent anthelmintic action in these cases.

Sheep.—Three sheep were given sodium fluoride in capsules or in the feed. One sheep weighing 115 lb. was given 15 Gm. of the drug in the feed. Inappetence and soft feces were observed the day after treatment, but both conditions showed gradual improvement. Diarrhea reappeared on the seventh day, and one day later the animal suddenly went off feed. The sheep was badly fly-blown on the ninth day and died during the night. Postmortem changes were extensive in all internal organs. Each of 2 sheep weighing 110 to 120 lb. was given 10 Gm. of the drug in capsules. Both animals had soft feces and were off feed the day after treatment. One sheep gradually improved and was apparently normal on the eighth day. The other animal, however, died on the ninth day, and areas of congestion and necrosis were found in the gastrointestinal tract at the necropsy.

Chickens.—Two chickens were given sodium fluoride as a 1.5 per cent mixture of the feed for ten consecutive days. Although no other feed was made available to them, the birds refused to eat an appreciable amount of the mixture and the droppings consisted largely of urates. There was no anthelmintic action, and congestion of the small intestine was observed in one bird at the necropsy.

Dogs.—Two dogs were given sodium fluoride in capsules or with the feed. One dog weighing 37 lb. was given the drug as a 1.0 per cent mixture of the feed. Vomition occurred about an hour after treatment, and the dog refused to eat more of the mixture. The appetite was not impaired, however, for regular feed was eaten when offered to the animal. Another dog weighing 24 lb. was given 1 Gm. of the drug in capsules after a fast of twenty-four hours. The animal vomited several times shortly afterward. Four days later and at successive intervals of four days, doses of 1.0, 1.5, 2.0, and 2.5 Gm., given in capsules after a regular feed, were retained with no observable toxic reactions. A dose of 3 Gm. administered in the same manner, however, induced profuse vomition accompanied by marked depression and inappetence. The dog was apparently normal

two days after treatment. There was no anthelmintic action, and the necropsy revealed a moderate hemorrhagic gastroenteritis.

DISCUSSION

When given to swine as a 1.0 per cent mixture of the feed for three consecutive days, sodium fluoride was very effective against large roundworms but of variable or insignificant efficacy against stomach worms, nodular worms, and whipworms. Although the ascaricidal action was quite promising under these conditions, it seemed desirable to eliminate, if possible, the plurality of feedings. Consequently, the drug was given to swine for one day as a 2.0 per cent mixture of the feed. With this procedure the ascaricidal action was maintained at a high level and, in addition, the action of the drug against stomach worms was apparently increased. Moreover, the reduction of sodium fluoride to 1.5 per cent of the feed for one day did not materially decrease the anthelmintic action against these parasites. A further reduction in the amount of the chemical to 1.0 per cent did not decrease the ascaricidal action, although at this level the efficacy against stomach worms could not be adequately determined from the number of cases at hand. The action of the drug against nodular worms and whipworms, however, did not change appreciably with alterations in the regimen of therapy; there was no evidence in any case of effective action against these parasites. This experience suggested the tentative conclusion that the administration of the chemical at a rate of 1.0 per cent of the feed for one day represented a reasonably close approximation to the optimum dosage.

It cannot be emphasized too strongly, however, that much further investigation will be necessary before sodium fluoride, however administered, can be endorsed as a safe and suitable anthelmintic.

Mixtures of sodium fluoride and phenothiazine were disappointing in that they were not uniformly effective in removing significant numbers of nodular worms, though the combination seemed to impart a markedly less palatable flavor to the feed than either substance alone. Attempts to mitigate this condition by incorporating various amounts of molasses or sour whey into the feed mixtures were to no avail. The ascaricidal action was complete in all instances, but the addition of sufficient phenothiazine for significant anthelmintic action against nodular worms apparently rendered the mixture markedly unpalatable

In view of the unfavorable publicity attributed to the toxic properties of sodium fluoride, other fluorides, *viz.*, aluminum silicofluoride, barium fluoride, aluminum fluoride, sodium aluminum fluoride, and sodium silicofluoride were tested in swine, and the latter two in sheep also. None of these compounds demonstrated anthelmintic action comparable to that of sodium fluoride. The virtual absence of anthelmintic action in these combinations is probably due, at least in part, to the lower percentages of available fluorine. Sodium fluoride at therapeutic levels, nevertheless, was surprisingly well tolerated by swine, the only untoward reactions being vomition in some of the animals, and, in a few instances, diarrhea or soft feces. Vomition, however, was unattended by nausea and depression and, therefore, cannot be regarded as an alarming reaction. Indeed, some pigs vomited shortly after consuming regular feed as well as after consuming medicated mixtures, but they continued to eat the mixtures almost without interruption even if vomition had been induced. It was observed, moreover, that when sodium fluoride was given to swine in the feed, at therapeutic levels, the pigs did not eat the mixtures in sufficient amounts to incite toxic reactions of greater severity than the mild reactions already described, such as occasional vomiting and soft feces. Indeed, this self-limited consumption of medicated feed seemed to afford some natural protection against intoxication. In virtually all instances, however, sufficient amounts of sodium fluoride were consumed and retained to produce complete, or nearly complete, ascaricidal action. The inappetence was only apparent, since regular feed was eaten readily. Although 4 pigs died during the therapeutic trials, necropsy findings indi-

cated that pneumonia was either the primary, or a highly contributory, cause of death in 3 cases, and in view of the heavy lungworm infections and poor condition of most of the test animals before treatment, the number of fatalities from extraneous causes was unexpectedly low.

The gross pathology, histopathology, and toxicology of acute and chronic fluorine poisoning have been fully reported in an extensive literature, including many textbooks. As a consequence, an exhaustive study of these features was not undertaken in the present report. It seemed desirable, however, to ascertain with some degree of accuracy the tolerance of swine and other animals for the compounds, and to observe the symptomatology of acute fluorine poisoning in the common farm animals. Such studies indicated that a 5.0 per cent mixture of sodium fluoride in the feed is fatal to swine. The syndrome of acute fluorine poisoning is vomition, apparent inappetence, depression, weakness, trembling, and death. The essential postmorten findings are hemorrhagic gastroenteritis and marked congestion of the liver and kidneys. Toxicity studies in horses, cattle, sheep, goats, chickens, and dogs showed that the symptomatology and gross pathology, consequents of the ingestion of sodium fluoride by these animals, are very similar to those observed in swine. Furthermore, these studies seemed to indicate that most farm animals, like swine, can withstand relatively large doses of sodium fluoride or that they refuse to eat or retain sufficient quantities to produce fatal consequences. Therefore, it appears unlikely that accidental poisonings of these animals would occur under ordinary circumstances if reasonable precautions were taken. However, in order to minimize accidental poisoning of man or animals, it is strongly recommended that only tinted sodium fluoride be procured for anthelmintic purposes, in the event the chemical in question should find general use as a vermicide.

SUMMARY

Anthelmintic and toxicity tests with sodium fluoride, sodium fluoride in combination with phenothiazine, and five other inorganic fluorides were carried out on 151 pigs, 7 goats, 5 sheep, 1 bull, 7 horses, 2 dogs, and 2 chickens.

In trials with sodium fluoride as an anthelmintic for hogs, tests were made with 128 pigs, mostly in very poor condition, to which the chemical was administered in dry feed, usually for a period of one day, at rates of 1.0, 1.5, 2.0, 4.0, and 5.0 per cent of the feed mixtures, the higher concentrations being employed chiefly to determine the tolerance of pigs for the chemical. Feed mixtures containing 1.0 per cent and 1.5 per cent of the chemical were fed for one day in group tests as well as in individual critical tests. In the several tests, the efficacy against ascarids ranged from 67.0 to 100 per cent and against stomach worms, from 76.0 to 100 per cent; the efficacy against nodular worms and whipworms was too low to be significant. These efficacies prevailed at the minimum dosage tested; namely, 1.0 per cent in the feed for one day. At this dosage, the compound removed an aggregate of 262 (97%) of 270 ascarids from 52 pigs. Among the pigs so treated, there were 2 deaths, 1 from pneumonia and 1 from undetermined causes, and 2 pigs that were in very poor condition on and following the third day after treatment. The other pigs showed no significant ill effects, in spite of the fact that the experimental pigs, as a lot, were bad risks for medication on account of their poor condition. The effective dosage was approximately 0.13 Gm. per pound of body weight, although the intake per animal probably varied considerably. In the specific toxicity tests, it was found that feed mixtures containing as much as 4.0 or 5.0 per cent of sodium fluoride were highly dangerous; of 2 pigs tested at each dosage, only 1 of those on the 4.0 per cent ration survived, although the other pig similarly treated showed pneumonic lesions *post mortem*.

In trials with 17 pigs, phenothiazine and sodium fluoride in ratios of 1, 1.4, 2, and 3 parts of the latter to 1 part of the former, were tested at rates of 1.2 to 2.0 per cent of the feed. These treatments were 100

per cent effective against ascarids, and were well tolerated. The efficacy against nodular worms was only 42.0 per cent, and feeds containing both chemicals in combination were unpalatable.

Other fluorides, *viz.*, aluminum silicofluoride, 2.0 per cent; barium fluoride, 3.0 per cent and 2.0 per cent for two days; aluminum fluoride, 1.5 and 3.0 per cent; sodium aluminum fluoride, 2.5, 5.0, and 10.0 per cent; and sodium silicofluoride, 1.0, 2.0, and 5.0 per cent, fed for one day except as mentioned, at the percentages indicated, showed from poor to moderate anthelmintic action or none at all.

To test the tolerance of other farm animals for sodium fluoride, the chemical was administered in sizable doses to 7 horses, 1 bull, 7 goats, 3 sheep, 2 chickens, and 2 dogs. The results indicated that this chemical was sufficiently well tolerated to preclude, for practical purposes, the possibility of accidentally poisoning stock under the same conditions in which it might be used as an anthelmintic for hogs. These tests suggested also that the chemical has some anthelmintic action against ascarids and oxyurids in horses.

In was concluded that sodium fluoride is promising as a medicament for the removal of large roundworms from swine, and that feeding it at the rate of 1.0 per cent in dry feed for one day approximated the optimum dosage. It seems possible that lower dosages might be equally efficacious and, at the same time, safer.

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Corrigendum

The first line in column 3 of table 4 (page 78 of the April issue) should read 7.85 (avg. 32 eyes) instead of 0.94 (avg. 22 horses).

Epizoölogy of Western Equine Type Encephalomyelitis: Eastern Nebraska Field Survey of 1943 with Isolation of the Virus from Mosquitoes

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THE VIRUS of western equine encephalomyelitis was isolated from horses in 1930[1] and from man in 1938[2] and was experimentally transmitted by laboratory infected Aedes mosquitoes in 1933.[3] It was first found in mosquitoes during the summer of 1941.[4,5] This isolation from mosquitoes was accomplished in the Yakima Valley, Wash., in the presence of an epidemic and epizoötic. Five isolations were made from *Culex tarsalis* Cop., a mosquito which had never received much attention previously, since it only occasionally bites man, and had never been suspected as a disease vector. During the same season, strong evidence was collected which implicated domestic fowl as important "reservoirs"* of the virus in this area.[6,7] The following year, while working in the same valley, experiments were begun which proved that *C. tarsalis* became infected if permitted to feed on inoculated ducks or chickens.[8] Some of these infected mosquitoes, if held eight to thirty days at high summer temperatures, were then shown to be capable, by their bite, of infecting other chickens. At no time did the infected fowl appear ill, but infection was detected by demonstrating the presence of the virus in the chicken's or duck's serum by the intracerebral inoculation of mice which subsequently developed typical encephalitis. During 1942, in the Yakima Valley, 44 isolations of western equine type virus were made from mosquitoes,[9] most of them, again, from *C. tarsalis.* During 1943, in Kern County, Calif., 31 isolations of western equine virus were made from mosquitoes,[10] 28 of the 31 isolations were from *C. tarsalis.* Studies of the feeding habits of this vector, in both areas,[11,10] showed that it fed principally on birds, next in order of choice was the cow, then the horse. Most of the infected mosquitoes were collected from or near chicken coops. Thus, in three successive years, in two widely separated, hot, irrigated valley areas, *C. tarsalis* was found infected repeatedly. As a further indication of the rôle played by fowl, it was found that from these epidemic and epizoötic areas 26 to 50 per cent of all chickens sampled by serum neutralization tests showed evidence of past infection with the western equine virus.[7,10] Howitt made similar surveys of chickens in the San Joaquin Valley, Calif.[12] and found evidence of widespread infection, 57 per cent positive where epizoötics occurred. As a result of these studies, it appeared well established that the fundamental biologic cycle of this virus, in the areas surveyed, was *fowl to mosquito to fowl,* and that occasionally an aberrant manifest infection occurred in horse or man through the bite of one of these infected mosquitoes. Virus is found so much more readily and in so much higher titer in the blood of chickens and ducks than in that of the horse, cow, or man[13] that these large mammals appear, at

The work reported here was carried out in collaboration with the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army; and under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development, and the University of California, aided by a grant from the National Foundation for Infantile Paralysis.

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*"Reservoir" animal is used here and elsewhere in this paper when in quotation in a restricted sense. All known vertebrate sources of these viruses are infectious for a short period of time only, thus are not reservoirs in the usual sense of an animal having a latent or chronic infection which may serve as a source of infection over a long period of time. No such a true reservoir for these viruses is yet known. Such a reservoir if it exists might be vertebrate or invertebrate.

present, to be of negligible importance as a source of mosquito infection.

Surveys were made in lower Texas,[14] and the Gila River Valley, Ariz.,[15] in the year 1942, following an epizoötic in each of these areas in 1941. During these two surveys, no infected arthropods were found, but equine infections were also lacking that year.

Thousands of biting arthropods, other than mosquitoes, including ticks, mites, fleas, lice, flies, kissing bugs, and bed bugs collected in epizoötic areas have been tested by us[5,10] and by others[16,17] with negative results. We have found only mosquitoes infected, to date; however, Kitselman and Grundmann[18] reported that kissing bugs (*Triatoma sanguisuga* LeConte) were infected in Kansas and also that they were capable of transmitting the disease to guinea pigs.

In the laboratory, it has been demonstrated that 13 species (3 genera) of mosquitos[8,19] and 1 species of tick (*Dermacentor andersoni* Stiles),[20] if experimentally infected, can transmit the virus.

In the summer of 1943, it seemed important to us, in view of this previous work done in irrigated valley areas, to determine whether equine epizoötics in dry farming country were also due to an aberrant tangent of a fowl to *C. tarsalis* to fowl cycle. Cox, Jellison, and Hughes[21] had found a prairie chicken and a deer infected in North Dakota in 1941 and McNutt and Packer[22] in Iowa had isolated the western equine virus from an infected hog. Furthermore, no neutralization test surveys in fowl or mammals similar to those made in irrigated areas had been reported from dry farming country.

NEBRASKA SURVEY OF 1943

Through the coöperation of Dr. J. W. Murdock, of the Lincoln office of the Bureau of Animal Industry, United States Department of Agriculture, we were kept notified of the reported cases of equine encephalomyelitis in Nebraska in the early summer of 1943. As a result of this information, in early August we began to collect mosquitoes and other arthropods, and blood serums from wild and domestic birds in eastern Nebraska where the largest number of cases of encephalomyelitis was occurring. There were no proved human cases, although there was an increased incidence of poliomyelitis. Eight human cases of unknown etiology, without paralysis, were tested for antibodies to western equine and St. Louis virus with negative results.

In table 1 is presented the list of 10,886 mosquitoes and 1,480 bed bugs (*Cimex lectularius* Linnaeus*) tested for virus. Western equine encephalomyelitis virus was isolated from 1 pool of 67 *C. tarsalis* out of a total of 631 caught. This is the first reported isolation of an encephalomyelitis virus from any source from the state of Nebraska. In addition to our identification of this virus, it was confirmed by Dr. Giltner of the Bureau of Animal Industry Laboratory, United States Department of Agriculture, in Washington. No virus was found in the 9,000 Aedes (principally *Aedes vexans* Meigen) or in the bed bugs (collected entirely in chicken houses). *C. tarsalis*, which was found infected in this region, is a common mosquito even in midwestern states, and its period of greatest seasonal activity in Washington[9,19] and Saskatchewan[23] has been shown to correlate

TABLE 1—Arthropods Tested by Animal Inoculation for the Presence of Encephalitis Virus

ARTHROPOD SPECIES	NUMBER TESTED	STRAINS OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS ISOLATED
Culex tarsalis	631	1
Culex pipiens and *restuans**	1,092	0
Culex salinarius	56	0
Aedes vexans	8,364	0
Aedes nigromaculis	623	0
Aedes triseriatus	25	0
Aedes trivittatus	18	0
Psorophora ciliata	10	0
Psorophora signipennis	26	0
Anopheles punctipennis	41	0
Cimex lectularius	1,480	0
Total	12,366	1

*Adult females of these two species were found to be inseparable on the basis of those characters previously advanced as diagnostic.

*We are indebted to Dr. R. L. Usinger, P. A. Sanitarian (R), U.S.P.H.S. for identification of this species as *Cimex lectularius* Linnaeus.

well with the epizoötic and epidemic periods.

Of those species of mosquitoes found in Nebraska,[24] *C. tarsalis*, *Culiseta inornata* (Williston), *Culiseta incidens* (Thomson), *A. vexans* (Meigen), *Aedes dorsalis* (Meigen) and *Aedes nigromaculis* (Ludlow) have been shown capable of transmitting western equine encephalomyelitis virus experimentally.[8,19]

In table 2 are presented the results of neutralization tests performed against 3 strains of encephalitis viruses on the serums of 91 mammals and birds from eastern Nebraska. It will be noted that in contrast to results obtained in the Yakima Valley[7] and the San Joaquin Valley[10,12] reviewed previously, no antibodies to the western equine virus were found in any of the blood serums from chickens, pheasants, or rabbits. These latter results are comparable to those which we obtained in the Texas survey of 1942,[14] and suggest, at least, the possibility that in open, dry farming country the virus may be adapted to some other hosts. It should be remembered that the virus has been isolated from a prairie chicken, deer, and hog. Extensive surveys of rodents made by groups of Canadian workers in Saskatchewan and Manitoba have thus far failed to obtain definite proof of infected vertebrate hosts (other than horses and man).[16,17] Further investigations along these lines are indicated.

TABLE 2—Results of Neutralization Tests Against Three Encephalitis Viruses on Blood Serums from 91 Domestic and Wild Animals

Animal	Western Equine Virus			St. Louis Virus			Eastern Equine Virus		
	Pos.	Ques.	Neg.	Pos.	Ques.	Neg.	Pos.	Ques.	Neg.
Chickens Hatched Before 1943	0	0	17	0	0	17	0	0	8
Chickens Hatched, 1943	0	0	20	0	0	20	0	0	19
Young Wild Pheasants	0	0	27	2	0	20	0	0	16
Wild Rabbits	0	0	4	0	0	4	0	0	4
Horses	*.	.	..	7	1	9	0	1	16
Cows	7	3	1	0	1	10	4†	2	5
Total	7	3	69	9	2	80	4†	3	68

*A number of bloods were tested from sick horses, some of them previously vaccinated. Unfortunately none of these could be satisfactorily interpreted. All were positive or questionable.

†In view of other data available, we interpret this neutralization of eastern equine virus by cow serum as nonspecific in character.

CONCLUSIONS

From previous studies made in hot irrigated valley areas in the West, it has been shown that western equine encephalomyelitis virus is transmitted primarily by *C. tarsalis* mosquitoes and that the mosquitoes acquire their virus principally from domestic fowl. In the present survey made in eastern Nebraska, the western type encephalomyelitis virus was isolated from *C. tarsalis* mosquitoes, and not from any other species. However, there was no evidence of past infection found in the blood of chickens or pheasants.

ACKNOWLEDGMENT

These studies were made possible by the coöperation and help of many persons and several organizations in Nebraska, principally the following: Dr. J. W. Murdock and Dr. A. G. Beagle, United States Bureau of Animal Industry; Drs. C. A. Selby, W. S. Petty, and Mr. L. O. Vose, Nebraska State Health Department; Dr. Frank Breed, Norden Laboratories; and Dr. H. D. Tate, Department of Entomology, University of Nebraska. The veterinarians contacted in these studies expressed continual interest and were most coöperative in reporting new cases.

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Vibrio Fetus in Sheep

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THERE HAVE been a number of published reports of the isolation of *Vibrio fetus* as the causative agent of abortion in ewes and undoubtedly there are others that have not been reported. Nevertheless, so little is definitely known as to the spread of the disease that publication of any further information on such outbreaks seems warranted, if only to focus attention on this agent as an economic factor in sheep production.

HISTORY OF 1944 OUTBREAKS

In the spring of 1944, two outbreaks of abortion in sheep were brought to the attention of the Wyoming Agricultural Experiment Station. Flock A contained 290 ewes of which about 250 had been raised on the place from a small group of lambs. The ranchhouse, sheds, and corrals were situated on the edge of a ravine through which a small creek ran. In addition to this source, water was supplied by a windmill and tank; the ground about this was very wet from frequent overflowing of the tank. The sheep ranged over a considerable area of typical sagebrush country cut up into gullies and ravines. At this time, most of the hollows contained water from recent snows, and the sheep also had access to water in an old gravel pit a mile away. From June to October, summer pasture was provided for dry ewes—old ewes without lambs due to various reasons, and young ewes—from outside sources, and they were run with the range flock.

On April 5, an aborted lamb was brought in, although the flock was due to lamb in May. The owner was not especially concerned, as he thought this ewe might have been injured by a cow. However, three years previously when the flock had been smaller, 11 lambs from a total of 60 were lost through abortion, so the ewe was isolated until April 8, when she broke loose and rejoined the flock. *V. fetus* was isolated from the lamb, but no further abortions were reported.

Flock B consisted of 700 ewes due to lamb April 9, and 500 ewes from another source that were due to lamb May 20. Different bucks had been used on each flock, although at this time the ewes were running together. When purchased as 2-year-olds the preceding summer, 200 of the 700 were without lambs. The sheep watered at a creek and several springs. A month before the trouble started, a load of hay containing a noticeable quantity of prickly lettuce was fed. The belief that this was the causative agent may account for the delay in seeking a definite diagnosis. Aid was not sought until several months after the abortions had started. At the outbreak, alfalfa and native hay were being fed and the ewes were just starting on cake. The original flock of 700 started aborting in January, three and one-half months before they were due to lamb; a few lambs were lost at first and then a number of abortions occurred simultaneously. The second flock, due to lamb after the first group and not cut out, aborted in regular series about the same time as the others. The aborted fetuses were 6 to 8 inches in length. On learning that *V. fetus* was responsible, the aborting ewes were separated and the others were moved to new bed grounds. However, the outbreak seemed to follow a natural course, and as late as May 10 weak lambs were still being dropped. In the 700-ewe flock, 400 aborted, and of the 500 in the second group, 250 aborted. In addition to the loss of these lambs, 25 ewes died from dystocia because of dry fetuses and from other causes attributed to the infection. Other ewes took months to recover with a resultant shrinkage in wool production. The

From the Wyoming Agricultural Experiment Station, Laramie.

Approved for publication by J. A. Hill, Director, Wyoming Agricultural Experiment Station, Laramie.

owner very conservatively estimated his loss from the disease at $3,500. In discussing the problem, it was learned that a neighboring rancher, in 1943, had lost 33 lambs by abortion in one night and had marketed 700 to 725 lambs from 1,000 to 1,200 ewes. In the present season, another nearby rancher obtained only 29 lambs from 127 ewes; one small flock of 29 to 30 ewes produced 1 live lamb only; and another flock of 900 to 1,000 ewes had resulted in a 50 per cent lamb crop. None of these losses had been brought to the attention of veterinarians.

RECOVERY OF VIBRIO FETUS

The lamb from flock A, presented two days after being aborted, was quite immature and had no wool at all. About 10 to 15 cc. of bloody fluid was present in the peritoneal cavity, and only about 1 cc. of thick, bloody fluid could be withdrawn from the abomasum. The lungs had not inflated, and they still resembled liver tissue.

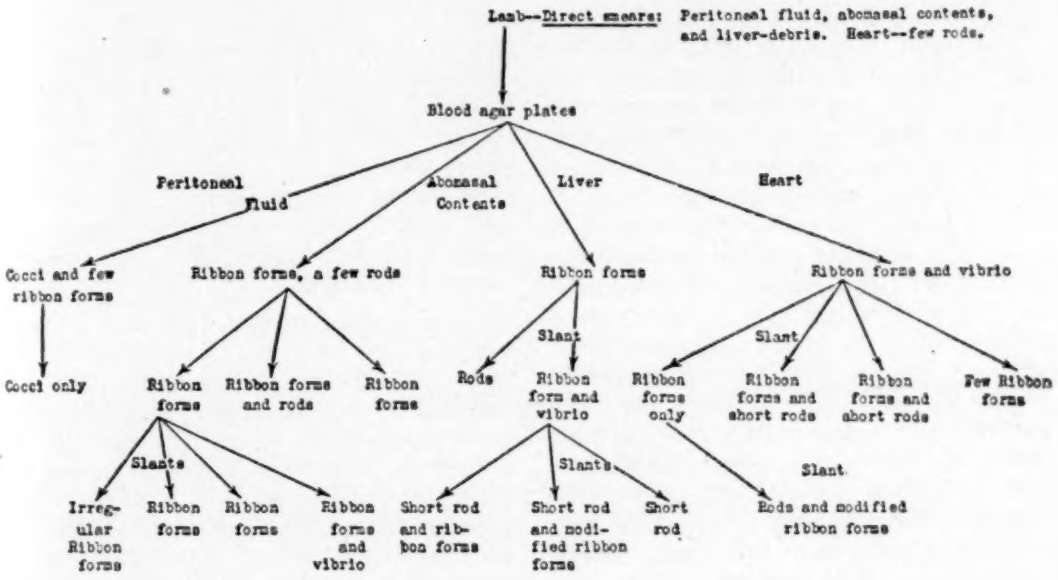
Direct smears of the liver tissue, stomach contents, and peritoneal fluid failed to reveal any organisms; moderate sized, straight rods were observed in a stained smear of heart muscle. Ten per cent sheep-blood, nutrient agar plates were inoculated and incubated for forty-eight hours in an atmosphere of 10 per cent CO_2. At this time, one of the plates streaked with contents from the abomasum presented slimy growth which, on microscopic examination, showed a peculiar organism: ribbon-like, with the peripheral outline taking the carbol-fuchsin stain distinctly, a lighter and sometimes irregularly staining interior, and a variably placed, heavier staining dot resembling a nucleus. Rarely, two of these bodies would be seen within the cell, at or near the poles. The size was usually about 1 μ by 10 to 15 μ. While some were straight, others were curved, and some were wavy. A similar organism was also responsible for minute amounts of slimy growth from the liver, heart, and peritoneal fluid. In the preparation from the heart, a few spiral-like filaments typical of *V. fetus* were found among the ribbon forms, and streptococcic colonies were present on the peritoneal fluid plate in addition to slimy material. Later transfers from these plates showed vibrios developing from the contents of the abomasum and heart. Growth was never abundant and 10 per cent blood-agar slants were employed without marked advantage. All incubations were carried out under 10 per cent CO_2, and reinoculations were made immediately after examining the cultures. The development of typical vibrios from pertinent cultures can be seen in table 1 in more detail. Later work has indicated that curved rods, spirals, and wavy filaments may not always show up, and single transfers may be predominantly short plump rods at times, so that the straight rods recorded may in reality in some cases be *V. fetus*. Table 1 approximately indicates the extent of cultivation of the organism; subsequent transfers were marked by more and more meager growth and recourse to the originals was of no avail.

Twin aborted lambs, secured from flock B, were not completely wooled out but were otherwise mature. Autopsy of lamb 1 revealed petechial and ecchymotic hemorrhages in the kidneys, about 50 cc. of egg-white-like material in the stomach, and the lungs were found to be partially expanded. Lamb 2 was partly covered by the fetal membranes; the abdominal cavity contained about 5 cc. of bloody fluid; the lungs were not expanded; and only 1 to 2 cc. of bloody material was found in the stomach. Stained smears of the contents of the abomasum, liver, and heart of the 2 lambs did not disclose any bacteria except for a few cocci from the liver of lamb 2. Cultural examination by means of blood agar plates incubated in 10 per cent CO_2 disclosed a delicate, grayish, slimy growth from liver, heart, spleen, and lungs of lamb 1 in which ribbon forms, as previously described, could be demonstrated. In addition, fusiform rods and the usual curved rods of *V. fetus* were isolated from liver and lung tissue. Grossly similar growth from the contents of the abomasum was made up entirely of vibrios. Results obtained by the inoculation of blood-agar slants were closely comparable. A second transfer onto blood-agar

plates resulted in pure cultures of vibrio forms from the contents of the heart, lungs, and abomasum. A third transfer from the contents of the abomasum, here-

a soil organism, no further effort was made to identify it. From trough water, handled in a similar manner, the same type of organism was recovered. No organism of the

TABLE I—Succeeding Generations of Cultures from Ranch A Source



Blood agar plates unless slant designated.

tofore, a straight culture of a typical vibrio, showed a few ribbon forms, otherwise there was a tendency for the ribbon forms to disappear, and the growth characteristics to assume more the appearance associated with *V. fetus*. Cultures of liver and heart of lamb 2 also contained these ribbon forms, but the peritoneal fluid was sterile.

EXPERIMENTS WITH MATERIAL FROM OUTBREAKS

Four hundred cc. of creek water from ranch A was centrifuged several hours at 1,500 r.p.m., and the sediment concentrated to 10 cc. This was streaked on blood-agar plates and handled the same as tissue cultures. Large rods with forms similar to the ribbon types described were isolated. However, they were larger, especially transversely, more granular, and more deeply stained. On further cultivation, the rods predominated. As this was considered nature of *V. fetus* or the ribbon forms associated with it were observed.

Twenty gallons of trough, creek, and gravel-pit water were brought from the same ranch. It was used as the sole water supply for one week (April 13 to 20, 1944) for 2 ewes bred February 2 and 12, respectively. Both ewes lambed normally.

A blood-agar slant and plate from lamb 1 of flock B, first transfers of cultures from abomasum contents, supplied a typical *V. fetus* culture for inoculation of 3 ewes. On April 6, 1 cc. of a No. 1 McFarland's nephelometer suspension was injected, intravenously, into ewe 216 (bred February 3); 3 cc. was given orally in a capsule to ewe 219 (bred February 7); and 3 drops was instilled in one eye of ewe 206 (bred February 2). Their temperatures were taken daily for the following week, and, except the temperature of 104.1 F. for ewe 216, the day following inoculation, they did not exceed

103.4 F. The external genitalia of ewe 216 were congested and slightly swollen on April 6, but the condition receded within the next two days. While no fetus was found at this time, this ewe failed to lamb, and these changes, although minor, would suggest an early abortion which escaped notice. Ewe 206 lambed normally June 25. As she failed to produce milk, the healthy lamb was raised by hand. An old, thin ewe to begin with, her condition became progressively worse and a slight vaginal discharge developed. She was killed on June 29; on autopsy, various lymph nodes were found enlarged; some fluid was present in the abdominal cavity and the pericardium; and the cervix was slightly congested. Cultures were made from the various tissues, but only those from the cervix and a cotyledon showed short rods, sometimes slightly curved. Neither the colony form nor the microscopic appearance was regarded as typical of *V. fetus*, and the cultures were not maintained. Later work with an established strain of *V. fetus* indicated that sometimes very large colonies may be obtained if they are isolated, and even the morphology may vary under different cultural conditions from the type usually associated with it. Ewe 219 had healthy twin lambs, on June 29. The last part of June, 2 visibly pregnant ewes were given 1/4 to 1/2 oz. of the stomach contents of lamb 1 from flock B, orally, in capsules after a small dose of copper sulfate solution. Both lambed normally within a week.

In retrospect, it is possible that the intravenous and ocular instillation of relatively small amounts of culture brought on infection. Although the stomach contents supplied the latter 2 ewes had been refrigerated, it is apparent that blood-agar plate cultures would not have been viable after three months of such treatment.

CULTURAL STUDIES

While the short viability of the isolations recorded here has not been typical of all cultures in our experience, Newsom[1] states that, in spite of using brain medium, his cultures also died out uniformly, within a short time. When, through the courtesy of Dr. H. W. Schoening, of the Agricultural Research Administration, United States Department of Agriculture, a luxuriantly growing culture of *V. fetus* in lyophilized form was obtained, the opportunity of studying some of its growth requirements was welcomed. This strain originated in sheep, having been obtained in 1942 from Dr. H. S. Cameron, University of California, and had been maintained on 10 per cent horse-serum agar or beef-infusion broth, incubated in an atmosphere of illuminating gas. Growth under aërobic conditions had been temporarily maintained for a short series of transfers.

Appreciating the fact that this culture might not be entirely typical of strains to be isolated, and as it was also the only one available to us at the time, no attempt was made to completely cover the subject. However, since so little is known in regard to the cultural requirements of *V. fetus*, which have largely remained unchanged since the earliest work with it, it was thought these limited observations might be useful to other investigators.

While the original report of MacFadyean and Stockman[2] has not been accessible to us, Smith[3,4] states that they cultivated their vibrio on a medium consisting of agar, gelatin, and serum in deep layer, as first used by Bang for *Brucella abortus*. Smith employed sealed agar slants, using a large inoculum of the original source material or adding defibrinated blood; serum and blood agar were also used; but the employment of the usual cultural mediums was unsuccessful. He also noted that individual cultures would vary in viability so that weekly transfers were recommended. In young cultures, the shorter forms predominated and his observation of the appearance of granules and other degenerative changes in older cultures has been repeated by others. He found the reaction of broth could be slightly on the alkaline side of phenolphthalein titration without interference with growth. Stockman[5] felt that alternation of broth with whole potato to potato agar aided the maintenance of cul-

tures, although Smith,[4] working with a number of strains, failed to secure growth on potato itself. Welch and Marsh[6] used brain-liver broth for their cultures. Curtis[7] has described a vibrio observed in pathological conditions of the human female genital tract which differs materially from *V. fetus* only in being a strict anaërobe. In ascites-agar tubes, numerous organisms were encountered in the water of condensation. Lee and Scrivner[8] successfully used sealed cystine-veal-infusion-dextrose agar slants and cystine-veal-infusion broth. Beyond Ryff's[9] description of Hammond's use of crystal violet veal-infusion agar plates in the isolation of *V. fetus*, no attempt has been made to develop a selective medium, although the material offered for examination is frequently such that overgrowth with contaminants, especially if slants are used, will result.

Our procedure was simply to try out different readily available or easily duplicated mediums in a search for one supplying optimum growth, with the idea that once such a medium were found, isolation would be simplified and rendered more certain, and cultures could be better maintained. With this practical aspect in mind, only the more common mediums and fundamental problems were approached.

Growth was readily obtained from the culture which had been lyophilized six months previously, suggesting the usefulness of this method in preserving cultures where the apparatus is available and good initial growth can be maintained. No difference could be detected between plates incubated in 10 per cent CO_2 and in a container from which the air had been exhausted and replaced by illuminating gas as suggested by Schoening.[10] As our containers available for the latter process were limited, and jars in the nature of tobacco humidors with the original covers or glass plates sealed with plasticine allowed the use of carbon dioxide, the CO_2 method was relied upon entirely. Carbon dioxide generated from calcium carbonate and hydrochloric acid was passed into a graduated flask over water, and 10 per cent of the volume of the incubating jar, without con-

TABLE 2

Serum (%)	Inoculum	Results	Blood (%)	Inoculum	Results
Series I	Plain Agar Base Medium. All Plates, Three Days 10 Per Cent CO_2, 37 C.				
3	suspension*	no growth	5	suspension	moderately heavy†
3	suspension	no growth	5	suspension	moderately heavy
5	suspension	3 colonies	10	suspension	heavy growth
5	suspension	1 colony	5	direct from culture	moderately heavy growth
10	suspension	light growth‡			
10	suspension	light growth			
5	direct from culture	15 colonies			
5	direct from culture	1 colony			
5	direct from culture	2 colonies			
Series II	Veal-Infusion Agar Base Medium				
3	suspension	no growth	5	suspension	moderately heavy growth
3	suspension	no growth			
5	suspension	no growth			
5	suspension	no growth			
10	suspension	no growth			
10	suspension	no growth			
Series III	Plain Agar Base Medium				
5	direct from culture	no growth	5	direct from culture	light growth
	1 Per Cent Tryptose Agar Base Medium				
5	direct from culture	3 colonies	5	direct from culture	light growth
	Veal-Infusion Agar Base Medium				
5	direct from culture	1 colony	5	direct from culture	few colonies

*Approximately No. 1 tube McFarland's nephelometer.
†Moderately heavy growth = confluent growth along all inoculation streaks.
‡Light growth = distinct separation into colonies along inoculation streaks.

sideration of the contents, was replaced by CO_2 by introducing a rubber tube near the bottom of the partially closed jar. Novy jars and bell jars with an opening at the top to admit the gas, placed on a glass sheet sealed with petrolatum, have also been utilized, so that this procedure should be adaptable to all laboratories.

In order to allow a quantitative aspect to the experiment with the mediums employed, pour plates were attempted by placing a definite amount of saline suspension of viable culture in a Petri dish, using variable amounts of serum, and then adding a measured quantity of various melted, agar mediums cooled to 45 C. Although tried several times with amounts of bovine serum up to 10 per cent of the total volume and with mediums that would support surface growth, the method was unsuccessful and had to be abandoned. With certain mediums and dye concentration, simply streaking a loop of culture taken from another plate allowed differentiation as to growth, poor growth, or no growth, but, as is readily seen, was not satisfactory for close measurement of the degree of growth. By making a suspension of the organism in saline equivalent to the No. 1 tube of McFarland's nephelometer and further diluting this suspension 1:10, 1:100, and 1:1,000, a certain degree of quantitative differentiation of plate mediums could be ascertained. A standard loop of each suspension was streaked a definite number of times on a sufficient number of plates and on 5.0 per cent sheep-blood, Difco's nutrient (1.5%), agar plates. By the use of this standard for each group, the necessity of standardizing the suspensions by count or other means was obviated.

With this culture or our previous isolations, no difference in growth was brought about with slant or plate methods. As the use of Petri dishes allowed a larger test surface and greater reliability in observing the results, they were used entirely. With the strain obtained from Schoening, no difference was noted if the agar plates were freshly made up or had been stored in the refrigerator for several days before use. All growth developing was examined microscopically as a check on identity, and, in addition, these hundreds of examinations allowed some generalizations: Grown on a suitable medium with a high percentage of blood, plump, short, straight rods with rounded ends predominated; where adverse conditions were encountered such as on dye plates, the number and length of filaments were increased and in some cases made up the entire growth; the typical curved rods and semispiral forms were best observed on 6.0 per cent agar, 5.0 per cent blood medium, although the growth was not great; while the usual stained preparation consisted of rather slender rods, sometimes in pairs, a variable number of which might be slightly curved, a number of short filaments usually not markedly curved, and in some cases a few short attempts at spiral formation.

EFFECT OF HYDROGEN ION CONCENTRATION

One of the first objectives was the determination of optimum pH for growth which, to our knowledge, has never been adequately investigated. In our first experiment, veal-infusion agar was adjusted to pH 5.5, 6.0, 6.5, 7.0, and 7.5 with the Coleman pH electrometer before adding 10.0 per cent sheep blood. Equally good growth was obtained from each series. With the idea that this amount of blood might have considerable buffering effect, a second series of adjusted mediums was made and only 3.0 per cent blood incorporated. Before the agar solidified, the pH was again determined and was found to have shifted 0.5 pH toward that for blood with one exception, so that the final readings were 5.1, 6.4, 6.6, 7.2, and 7.5. It might be that at a greater interval a more pronounced change might be detected. After inoculation and incubation, comparable growth was obtained with the plates in each series except for that of pH 5.1 which alone of the group had included a commercial tablet for buffering water (pH 5.0). With the pH 5.0 set, dilute suspensions as inoculums resulted in sterile plates, as did heavy inoculation with direct smears of culture with the exception of one plate which had a few colonies only. As

this strain grew well aërobically in liquid mediums without the addition of blood, Difco veal-infusion medium was adjusted to pH 5.0, 6.0, 7.0, 8.0, and 9.0 in lots of ten tubes. After inoculation and incubation, these were streaked on blood agar which on subsequent incubation showed growth only from the pH 6.0, 7.0, and 8.0 series. There was heavy growth from all the pH 7.0 tubes, lighter growth resulted from the pH 6.0 group, and frequently only a few colonies developed from the pH 8.0 inoculations. This would suggest that the pH requirements do not differ markedly from other common bacteria, and that, unless strongly buffered, the incorporation of blood in the mediums would tend to adjust it automatically. Veal-infusion medium (Difco, pH 6.8) was made with agar and salt without adjusting the pH and allowed as good growth on blood plates as when it had been adjusted to pH 7.0-7.4.

AMOUNT OF BLOOD OR SERUM REQUIRED

It should again be emphasized that only one strain was available, and that it had become markedly adjusted to artificial conditions. In testing it on different basic mediums, it has been customary to include the medium, unenriched with blood, in the series. In one such trial, growth followed heavy inoculation of plain nutrient agar. Incorporation of 1.0, 2.0, and 3.0 per cent blood in plain agar or veal-infusion base allowed adequate and indistinguishable growth, although slightly better results could be discerned with the inclusion of 5.0 per cent, or preferably 10.0 per cent, defibrinated, sheep blood. The marked superiority of defibrinated blood over serum is well illustrated in table 2. No difference was noted between cow and sheep blood.

SELECTIVE AGENTS

It was found by streaking suspensions of *Escherichia coli* and *Staphylococcus aureus* on plates containing various concentrations of brilliant green and crystal violet, with and without the addition of 10.0 per cent blood, that the presence of the latter decreased the inhibitory effect of the dyes. While the isolation of *V. fetus* would require the addition of blood, some selective action would be obtained by the inclusion of dyes, but their effect would not be as great as if used in unenriched mediums. With the inclusion of 10.0 per cent blood, streaking of *V. fetus* suspensions on 1:500,000 crystal violet agar allowed their growth, as on 1:100,000 and 1:500,000 brilliant green agar. With direct inoculation from culture, good growth appeared on 1:500,000 crystal violet 10.0 per cent blood-agar-plates and on 1:100,000 brilliant green 10.0 per cent blood agar, but only a few colonies developed from the inoculation of 1:100,000 crystal violet 10.0 per cent blood agar. With 3.0 per cent blood-agar plates, 1:500,000 crystal violet allowed growth from culture and suspension inoculations, but stronger concentrations of the dye—1:100,000 and below—resulted in sterile plates. With brilliant green under these conditions, 1:500,000 and 1:100,000 concentrations allowed growth but 1:10,000 dilutions of the dye checked it entirely. Hayward and Miles[11] have suggested making up nutrient agar with 6.0 per cent agar content to prevent swarming of Proteus. Such a medium with 10.0 per cent blood allowed growth indistinguishable from the control plates, but with reduction of the blood content to 5.0 per cent the colony size became very minute. However, the morphology was then made up almost entirely of curved rods and spirals. A disadvantage of this high agar content was that a uniform mixture of the blood with the cooled medium was obtained with difficulty.

USE OF DIFFERENT MEDIUMS

Difco veal-infusion medium with added salt and agar was possibly superior to plain nutrient agar for the growth of this strain, although the inclusion of blood tended to obscure any differentiation if the medium was at all satisfactory. One per cent tryptose agar, Difco tryptone glucose extract agar, and Difco brain-veal agar were in this category. Only heavy inoculations developed growth on Brewer's anaërobic agar with 10.0 per cent blood. Difco potato-infusion agar with added blood did not

support growth, as was the case with some of the mediums used in water analysis. Growth occurred in nutrient broth, but better development was obtained by the addition of 1.0 per cent tryptose and in Difco veal-infusion medium. Thioglycollate broth* was superior to these. Growth did not always follow the inoculation of cooked meat medium, but when it was established the viability of the culture approximated that in Hibler's medium.

*Neopeptone	10 Gm.
K_2HPO_4	2 Gm.
Sodium thioglycollate	1 Gm.
Agar	1 Gm.
Gelatin	1 Gm.
Meat extract	3 Gm.
Water	1 liter

Adjusted to pH 7.0.

INVOLUTION FORMS

Although this strain grew uniformly well and showed degenerative changes only in old cultures, when checking the viability of a Hibler-medium culture which had been incubated aërobically three days, held in the refrigerator four days, and then streaked on blood agar, a stained film of the confluent colonies that developed disclosed the same involution form as described for the natural outbreaks. These ribbon forms were comparatively few in number and the majority of the organisms seen were of the usual type. Prior to this inoculation the culture had been maintained chiefly on blood agar. Other tubes similarly handled remained normal, and attempts to maintain the variant were unsuccessful. One tube of veal-infusion medium incubated at 37 C. for five days and held at room temperature four more days before streaking on blood agar disclosed forms with marked granularity, greater than was associated with much older cultures.

Several procedures were followed in an attempt to produce involution forms. Blood from a sheep injected with this culture was used for making blood agar, but the agar produced normal growth. From 1.0 to 2.5 cc. of Seitz filtrates of old cultures were included in blood-agar plates just before solidifying without result. Five-grain sulfanilamide and 0.5-Gm. sulfathiazole and sulfamerazine tablets were placed in the center of blood-agar plates before hardening and the surface flooded with *V. fetus* suspension. On incubation, no inhibition was detected, and stained films taken near the periphery of the tablets failed to disclose any abnormality. Films prepared from dye plates and pH studies were also negative. Not enough growth of these ribbon forms was secured from any of the strains to permit serological or other studies, and the small amount available was sacrificed in unsuccessful attempts to propagate it.

DISCUSSION

The experience with flock A would indicate that prompt removal, even for a short period of time, of the first ewes aborting in a flock will serve to stop the infection, and that the discharges from such a ewe rather than the water supply is the infective agent. While this is but a single instance and our experimental work with the water supply here is limited, all accounts of brucellosis in cattle, in which the causative agent is comparably delicate, would substantiate it. The area involved near the B outbreak may be exceptional, but it will illustrate the prevalence of such a disease without the knowledge of the recognized authorities. Our experience with the size and handling of western ranches would not strongly suggest a common source of the infection in this locality.

If one were permitted to generalize on such meager data, the dry ewes pastured during the summer would seem the logical source of infection for flock A, for these older ewes were without lambs because they aborted or lost their lambs through other causes. One or a few of these were able to infect 1 ewe in the permanent flock which harbored the infection until well along in pregnancy when abortion occurred. One could attempt to explain this condition either by a partial or weak immunity in the majority of ewes due to the previous outbreak, to the small number of organisms present, or to their decreased virulence so that only 1 ewe so exposed developed

infection. This existed in latent form until the fetal membranes of pregnancy were involved sufficiently to result in premature expulsion of the fetus. Whether her isolation prevented spread of the infection to other ewes would then be dependent upon the virulence of the organism present in the discharges and upon the immunity retained by the other ewes. In flock B, these ewes from unknown sources might also have harbored a latent infection, or the increasing incidence of abortions from a few initially could well have occurred by the spread of *V. fetus* from the earlier aborting ewes. One thing is certain: Sheepmen will disregard a few abortions in the hope that more will not occur, and will not report a high incidence and risk the stigma and possible rigid quarantine unless effective control measures can be supplied by the veterinarian.

Stockman[5] has reported that the disease is sporadic, tends to occur only on certain farms, and frequently disappears only to reappear again in a few years. Smith,[12] from his observation that the disease practically ceased in older cattle until younger animals were introduced, concluded that the infection tended to die out because herd immunity developed and persisted unless a more virulent strain of Vibrio were introduced. In a brief footnote to the Graham and Thorp[13] report of abortion in sheep, the disease is described as appearing the following year on the same premises. However, they were able to obtain experimental animals from the owners because they desired to dispose of them. No explanation as to the conditions of the second outbreak, whether or not new sheep were introduced to remnants of the old flock, is given. Baker and Stone[14] held over 8 ewes that had aborted in an infected flock to find they lambed normally the following year. Ryff[15] also noting that the infection may have originated from a neighboring, aborting flock, found the same to hold true with a larger group held over under the same conditions on the same farm. Seven primigravid ewes lambed normally in this flock the year following the outbreak, but they were not new additions. Gilman[16] reported four abortions in 27 imported ewes added to a flock of 50. The following year 2 other ewes from this source, a ewe native to the flock and another addition, aborted, and *V. fetus* was isolated from three of their fetuses. One interpretation of this would be that the disease is very much like brucellosis in cattle, in which the tendency is for affected animals to abort only once, and that frequently it is only when a large influx of new animals is exposed that attention is again drawn to an outstanding number of abortions.

Stockman[5] reported an interval of thirteen to one hundred thirteen days, and the work of Lee and Scrivner[8] would indicate ten to twenty days, but a period of even up to seventy-two days might intervene between experimental inoculation and abortion. This does not answer the question whether a series of abortions are due to an initial infection about the same time in the past or chiefly to rapid spread from animal to animal at the time of abortion. The incidence of an infectious disease in a susceptible group tends to start with a few cases, followed by a marked acceleration, and then a gradual tapering off; this is a description that usually applies to an outbreak of vibrionic abortion in sheep. Smith's[3,17] work with cattle indicates that it is essentially a disease of the genital tract with secondary involvement of the fetus, and it is possible that, like bovine brucellosis, a quiescent infection becomes apparent only in pregnancy and may then spread rapidly to other susceptible animals. Gilman's report,[16] in which the infection is attributed to imported ewes, would tend to substantiate this, although this outbreak involving only a few animals and spread over two lambing seasons also suggests an organism of low virulence.

Enough work has been done to incriminate *V. fetus* as a cause of abortion in ewes, although the uncertainty of infection following experimental oral inoculation would indicate a variation in susceptibility, and attention should be directed to the primary source of infection. On the range where stagnant pools may have to be de-

pended upon for water, this agent is frequently blamed and perhaps justifiably so. It would be our opinion that *V. fetus* is too delicate an organism, as it occurs in animal tissues, to exist as a saprophyte, although undoubtedly such conditions would temporarily accelerate the spread of the disease if the water became contaminated. The experience of flock A with ample access to stagnant water would substantiate this unless an outstanding immunity is attributed to the flock.

Our first observation of the variant described left us checking various sources with the thought that it might be a contaminant and even considering the possibility of a carry-over of an embryonic tissue form or a protozoan, so that its dying out in the first isolation was dismissed in view of its implied complexities. Finding it in a second outbreak and again in a regularly growing strain, temporarily subjected to new growth conditions, strongly indicated its variant nature, even though attempts to obtain it again failed. Lack of a Vibrio organism in films of aborted material should not discourage further attempts to demonstrate it culturally, and even atypical forms should be regarded with suspicion until further cultural work can rule them out. Our own efforts have indicated a great difference in ease of cultivation in different strains. Before recommending any definite procedure or mediums, it would be well to apply it to a number of isolations over a period of time, but it is hoped these observations made with one strain will stimulate further work with isolations of other workers.

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Control of Intestinal Parasitism in Lambs by Winter Treatment of Ewes, as Compared with the Use of Phenothiazine in Salt in Summer

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IN CONNECTION with the investigation of the relation of intestinal parasites to the health of range sheep in eastern Montana, it has become necessary to obtain information on the effectiveness of anthelmintics against the nematode parasites found in sheep in that area, and under the conditions of sheep-raising in the area. The many published reports on the subject of control of intestinal parasitism in sheep do not seem to cover adequately the situation in eastern Montana. This is partly because the reports of experimental work on control of intestinal parasites of sheep, particularly in the United States, have been concerned principally with *Haemonchus contortus* and *Oesophagostomum columbianum*. In the northwestern states, the nodular worm is not found, and, while Haemonchus occurs to some extent, it is not of major importance in the northern Great Plains area. The principal nematode parasites present in the abomasum and small intestine of sheep in this region are species of Ostertagia, Nematodirus, and Trichostrongylus.

While the significance of intestinal parasites in range sheep on the northern plains is not yet clear, there is reason to suspect that Trichostrongylus and possibly Nematodirus are factors in the diarrhea that affects many lambs in the area at the age of 3 to 4 months. The general presence of Moniezia in lambs in that area may also be a factor, and further investigation of the possible pathogenic effect of this cestode is needed.

As treatment directed at Moniezia had not always been successful, the sheep owners were advised to try control measures for the trichostrongylid worms. Since published reports indicate that phenothiazine is the most effective anthelmintic against the intestinal nematode worms now available, the use of phenothiazine was recommended in War Circular 5, published in October, 1943, by the Montana Veterinary Research Laboratory, for range sheepmen. Because treatment of lambs after the diarrhea develops is not as satisfactory as prevention, it was recommended to treat the ewes in the late fall after freezing weather begins, assuming that a large part of the winter carry-over is in the ewes rather than on the range. As insufficient information was available for the plains area on the effectiveness of this procedure, an experiment was set up at the station at Bozeman, to determine the results of ewe treatment as measured in the lambs.

METHODS

The plan of the experiment was as follows: The flock of 110 breeding ewes which is maintained at the veterinary laboratory was divided into 2 lots of 55 each on Jan. 18, 1944. Lot 1 was to be treated with phenothiazine once at the beginning of the experiment, while lot 2 was to be left untreated. Because the one treatment failed to result in maintaining a low, fecal egg count until lambing time, two additional treatments were used. Starting April 6, salt containing 10 per cent of phenothiazine was made available to both lots at all times. After lambing, the sheep were moved to summer pasture, on which there had been no sheep since Nov. 26, 1943. The pasture was divided, one portion to be grazed by lot 1, and the other by lot 2. Phenothiazine salt was supplied in both pastures. The results were measured in terms of egg counts on fecal samples from the lambs, with the object of determining whether the winter treatment of the ewes would reduce the parasite infection in the lambs under the condition that phenothiazine salt is taken in the spring and summer.

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The sheep used were grade Rambouillet ewes, produced at the laboratory. During the winter they were held in small pastures and fed hay. Sheep had been in these small winter pastures during the fall, and therefore, there had been opportunity for the deposit of worm eggs on the ground up to the time the experiment was started. At the time of the first treatment, the ground had been covered with snow since Dec. 26, 1943, and on January 24, two days after treatment, two inches of snow fell, and the maximum daily temperature was below freezing for six days. Four inches of snow fell again on February 4, and from February 9 to February 22, the maximum temperature was never above freezing, and snow fell several times. During the last week in February, there were several days when much of the small pasture in which lot 1 was held was bare and grazed to some extent. After April 1, the sheep were held in dry corrals, and fed hay from racks, which should minimize the pick-up of parasite larvae, during the period when the temperature was high enough to permit the hatching of eggs and the development of infective larvae. After lambing, the ewes and lambs were moved to the summer pasture.

The summer pasture was an irrigated bluegrass pasture of about 30 acres. It had been grazed by sheep during the summers of 1942 and 1943. No sheep had been on the pasture since Nov. 26, 1943. On April 5, 1944, sod samples were removed from an area on the pasture where sheep had bedded in 1943, and placed in a Baermann apparatus for forty-eight hours. Infective larvae of Ostertagia, Trichostrongylus, and Nematodirus were identified from this material. The numbers were small, being in the minority as compared with free-living soil nematodes. The carry-over of parasites on the pasture was also checked by fencing off a small plot and placing worm-free lambs on the plot. On June 5, three worm-free lambs were placed on this plot. They were removed June 19, and held in a pen on wire. On July 6, fecal examinations showed the presence of Ostertagia and Nematodirus eggs. On July 24, one of the lambs was killed to determine the worm content. Seventy Ostertagia, 100 Nematodirus, and no Trichostrongylus were found.

The results of the treatment with phenothiazine were measured by fecal egg counts on both ewes and lambs. The method used was a modification of the Lane centrifugal flotation method, as described by Stoll,[1] the principal difference being in the use of a sugar solution, 100 Gm. sucrose to 100 cc. water, instead of salt solution, for the flotation medium. Ten-Gm. samples were used, collected from the rectum of each sheep with the aid of a wooden spoon constructed for the purpose.

The trichostrongylid eggs were recorded as a group, with the exception of Nematodirus, which was recorded separately. Those recorded as a group include Haemonchus, Ostertagia, and Trichostrongylus. It can safely be assumed that very few of the eggs counted were Haemonchus. Although no attempt was made to make an exact differential count, it was noted that the eggs were predominantly of the Trichostrongylus and Ostertagia types, and this flock of sheep has always been relatively free of Haemonchus. The fact that the egg counts of the treated and untreated ewes were practically the same in May, also indicates that Haemonchus was not a factor, as phenothiazine is known to be very effective for Haemonchus, and the winter temperatures were low enough to prevent carry-over on the pasture.

Egg counts made to check the immediate result of treatment were made five days after treatment, as previous experience had shown that the minimum egg count was not reached until that time.

From April 6, at the beginning of lambing, until October 9, when the experiment was terminated, all the sheep had access to salt containing 10 per cent of phenothiazine. The salt was fed in boxes which were covered in such a way as to protect the phenothiazine from direct sunlight. The amount placed in the boxes was approximately what would be consumed in one week. The consumption of phenothiazine by the 111 ewes and their lambs was 65 lb. The number of ewe-days was 19,299, which divided into 65 lb. (29,510 Gm.) gives an average daily intake of phenothiazine of 1.5 Gm. per ewe and her lamb. There were 110 lambs in the experiment. Adding the number of lamb-days, calculated from the birth of the lamb to the end of the experiment, to the number of ewe-days, gives a total of 36,238 sheep-days. Dividing this figure into 29,510, the number of grams of phenothiazine consumed, gives an average daily consumption of 0.8 Gm. per head. The actual consumption was greater than 0.8 Gm. for the ewes and for the older lambs, as the consumption by lambs during the first month must be much less. According to Shorb and Habermann,[2] 0.5 Gm. of phenothiazine per day will completely inhibit development of nematode eggs in feces.

RESULTS

Trichostrongylus, Ostertagia, and Haemonchus in the Ewes.—On Jan. 18, 1944, fecal egg counts on the 110 ewes in the experiment showed an average of 120 eggs per gram for lot 1, with a range of 0 to 1,120, and an average of 115 for lot 2, with a range of 0 to 1,150. On January 22 the ewes in lot 1 were treated with phenothiazine, using the standard dose of 25 Gm. Commercial preparations of phenothiazine

such as are available to sheepmen were used. The ewes in lot 2 were not treated. On January 27, five days after treatment, fecal samples showed an average count of 12 for lot 1, with a range of 0 to 90, and of 80 for lot 2, with a range of 0 to 550. Fecal samples taken on February 23 showed no significant change in egg count, the average for lot 1 being 20 and for lot 2 being 75. On March 23, the average fecal counts for both lots were higher than before treatment. Lot 1 averaged 125, with a range of 0 to 635, while lot 2 averaged 230, with a range of 0 to 1,200.

Because of the increase in egg count, the ewes in lot 1 were again treated with phenothiazine on March 31. The reduction in egg count, as determined from samples taken five days after treatment, was less than 50 per cent, the average count being 70, with a range of 0 to 465.

The first lambs were born on March 31 and the last lamb came on June 14. The extended lambing period complicated the experiment somewhat, but was the result of having used the same ewes in work with another project. During lambing, the ewes were fed in corrals from racks, which should minimize the ingestion of worm larvae as compared with grazing.

The ewes were moved to the summer pasture in several groups between May 3 and June 19. Just before each group was moved to the pasture, fecal egg counts were made, and the ewes of lot 1 were again treated with phenothiazine. The average counts of the two lots before treatment were practically the same, and higher than they had been at any time before. Lot 1 averaged 535, with a range of 0 to 4,290, and lot 2 averaged 590, with a range of 0 to 3,030. The average count for the ewes of lot 1 five days after the final treatment was only 10 eggs per gram, with a range of 0 to 80.

Fecal counts for the ewes were made again on August 7, when the averages for the two lots were practically the same. The average count for lot 1 was 47, with a range of 0 to 395, while the average for lot 2 was 55, varying from 0 to 1,550. Eliminating the one high count of 1,550, the average was 22.

Nematodirus in the Ewes.—On January 18, the average fecal count of Nematodirus eggs for lot 1 was 9, with a range of 0

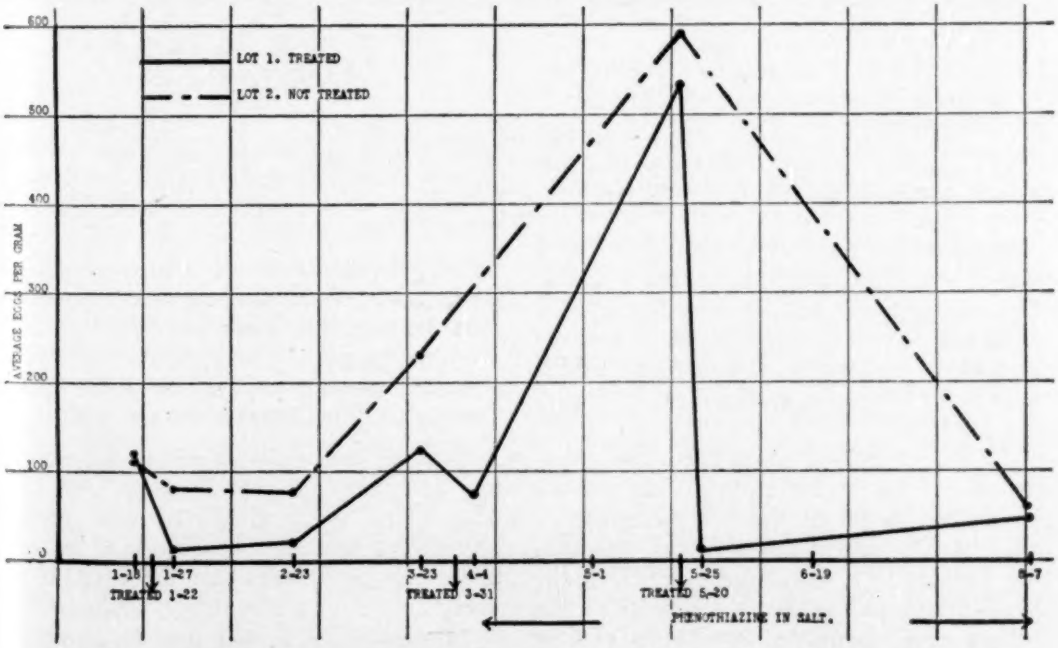

Chart 1—Average fecal egg counts for Trichostrongylus, Ostertagia, and Haemonchus of the ewes of lot 1, treated three times with phenothiazine, and of lot 2, not treated. Both lots received phenothiazine in salt.

to 200; while in lot 2, it was 4, with a range of 0 to 80. Treatment apparently had little effect, as both lots showed a reduction of about the same percentage on January 22. On February 23, the number of Nematodirus eggs was so low in both lots as to be of no significance. On March 23, there was a slight increase, to 7 for lot 1 and 8 for lot 2. Following treatment of lot 1 on March 31, there was no drop in egg count. The counts taken just before going to summer pasture averaged 35 for lot 1, and 13 for lot 2. After treatment, lot 1 averaged 18, a drop of 50 per cent. On August 7, the Nematodirus count for lot 1 averaged 2, and for lot 2 averaged less than 1, with only 1 sample of 53 showing any Nematodirus eggs.

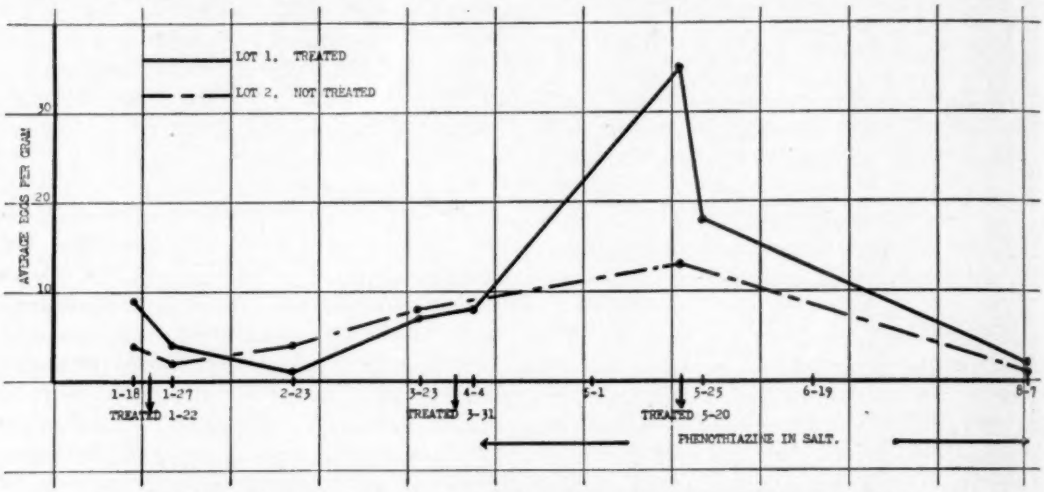

Chart 2—Average fecal egg counts for Nematodirus of the ewes of lot 1 and lot 2.

The average fecal egg counts for the 2 lots of ewes are shown in charts 1 and 2. The charts show the averages of counts made just before the sheep went to summer pasture, as of May 20, although the counts were made in several groups between May 1 and June 19.

As the effects of parasitism are manifested in the lambs rather than in the ewes, the results of the control measures used have been evaluated by the effect on the lambs. This was measured principally by fecal egg counts, and secondarily by the presence or absence of symptoms and by the weights attained by the lambs.

The first egg counts for the lambs were made when they were from 2 to 3 months old. The first counts for the May lambs were made on July 5, and for the June lambs on August 7. All the lambs were sampled on August 7, September 11, and October 9. There was no significant difference between the egg counts for the lambs of the two lots at any time, and the average counts for Ostertagia, Trichostrongylus, and Haemonchus were all below 100, except the first count of the lambs of lot 1, which was 125. The following tabulation shows the results of the egg counts.

	Lot 1				Lot 2			
Date	No.	Av.	Min.	Max.	No.	Av.	Min.	Max.
7- 5	26	125	5	365	26	90	0	465
8- 7	52	55	0	590	54	45	0	325
9-11	52	70	0	575	55	90	5	455
10- 9	52	25	0	205	55	100	5	1025

The Nematodirus egg counts were relatively high in the lambs, with no significant difference between the two lots. The averages ranged from 30 to 80, with Nematodirus eggs showing in all the lambs. The highest individual count was 345.

None of the lambs in either lot showed any symptoms of parasitism at any time during the summer. There was marked absence of any sign of diarrhea, and the lambs showed "bloom" on the wool. While gross evidence of parasitism has been rare in the veterinary flock in past years, during the summer of 1943, 11 lambs on the same pasture used in 1944 developed diarrhea,

which was correlated with a relatively high nematode egg count.

On Oct. 9, 1944, 38 lambs of lot 1, born between April 16 and May 27, averaged 75 lb. in weight. On the same date, 40 lambs of lot 2, born between April 17 and May 28, averaged 70.5 lb. In view of the fact that there had been no significant difference in egg counts on the lots, it must be concluded that this difference in weight is due to other factors than differences in parasite load. The average weight of the 2 lots combined was 72.6 lb., which may be compared with an average of 63 lb. for 39 lambs born between corresponding dates in 1943, out of the same ewes, grazed on the same pasture, but salted without phenothiazine, and weighed on Oct. 6, 1943. The fecal egg count on the 1943 lambs was 1,440 on October 6.

DISCUSSION

The results of this experiment are interpreted as indicating that winter treatment with phenothiazine of ewes not showing clinical parasitism is not profitable on a range where sheep will take salt in the summer.

Several conditions of this experiment must be considered in making a general application of the results to the control of parasites in range sheep. First, the parasite load in the experimental ewes was low at the beginning of the experiment. This condition, however, is probably rather general in ewes on the northern plains. Second, the only places available for holding the sheep during the winter were small pastures in which sheep had been grazed during the preceding summer and autumn. Therefore, the sheep were probably exposed to infective larvae during periods when they grazed to some extent. As noted above, from the start of the experiment to the beginning of lambing, the pasture was covered with snow most of the time, but there were periods when some grass was exposed, and at these times the sheep grazed somewhat. It is assumed that any pick-up of larvae during this period involved only larvae that had reached the infective stage before December, as temperatures during the winter months were too low for the hatching of eggs. The maximum daily temperature was never above 51 F. between December 4 and April 1, and the minimum daily temperature between those dates was never above 32 F., except for three days in March when the highest it reached was 38 F. The third consideration is that, even on ranges where the sheep take salt freely, it is much more difficult to attain the desired condition of each sheep taking salt daily than it is in the case of a small number of sheep in a farm pasture. It is also recognized that there are large areas in the range country where sheep take very little salt.

The graphs of chart 1 show that there was no increase in egg count in either lot between the treatment date of January 22 and the next sampling on February 23, but on March 23, the egg count of both lots was higher than when the experiment started. The count for the treated lot was, however, only half that of the untreated lot. This increase in egg count may be partially due to ingestion of infective larvae during a period late in February when the pasture was partially freed of snow. Lot 1 was given a second treatment with phenothiazine on March 31, which produced only 50 per cent reduction in egg count. Starting April 6, all the sheep were salted with salt containing 10 per cent phenothiazine, and were held in corrals under conditions considered unfavorable for extensive ingestion of larvae, but there was a marked increase in egg count in both lots up to the time they were turned out on summer pasture. This increase may be partly due to ingestion of infective larvae which survived the winter, and partly due to the ineffectiveness of the anthelmintic against immature Trichostrongylus. A third phenothiazine treatment at the time the sheep were moved to summer pasture reduced the egg count of lot 1 to an average of only 10 eggs per gram. Lot 1, then, was turned out on the summer pasture practically free from parasites as indicated by the egg count, while lot 2 went on pasture carrying enough parasites to produce an average egg count of 590 per gram. The

sheep were turned out in several groups between May 1 and June 19, and on August 7 egg counts for both lots were low and almost identical—45 for lot 1 and 55 for lot 2. The obvious conclusion from this unexpected situation is that the phenothiazine in the salt not only prevented reinfection, but also reduced the egg production, and possibly the worm population, in the untreated sheep to the same level as in the treated lot. The egg counts on the lambs also indicate that the phenothiazine in the salt equalized the two lots of ewes as far as being sources of infection to the lambs was concerned.

The results as to Nematodirus egg counts are interesting. The ewe counts indicate that the Nematodirus egg production, and possibly the number of worms, was reduced by the consumption of the phenothiazine salt. On the other hand, the lamb counts indicate that the development of the larvae was not inhibited in the feces, as the egg counts remained relatively high all summer, higher than the counts in similar lambs on the same pasture in 1943.

SUMMARY OF RESULTS

1) Two winter treatments of ewes with phenothiazine on January 22 and March 31 failed to result in the treated ewes having a lower load of Trichostrongylus, Ostertagia, and Nematodirus in May than the untreated controls, as indicated by fecal egg counts.

2) Ten per cent phenothiazine in salt, as indicated by egg counts on August 7, equalized the worm population of two lots of ewes, one of which went to pasture in May with an egg count of 590 per gram, while the other went to pasture with an egg count of 10 per gram, as the result of a third treatment.

3) The egg counts of Trichostrongylus and Ostertagia in the lambs of these two lots of ewes remained practically equal, and the average counts never exceeded 125 per gram. Lambs out of the same ewes on the same pasture in 1943 averaged 1,300 eggs per gram on August 7, and 1,440 on October 6.

4) Phenothiazine in salt apparently failed to inhibit the development of Nematodirus larvae in feces.

5) On Oct. 9, 1944, the average weight of 78 lambs born between April 16 and May 27 was 72.6 lb., while on Oct. 6, 1943, the average weight of 39 lambs born between corresponding dates, out of the same ewes, and on the same pasture, but not salted with phenothiazine salt, was 63.0 lb.

CONCLUSIONS

1) Winter treatment with phenothiazine of ewes carrying subclinical numbers of Trichostrongylus, Ostertagia, and Nematodirus, will not pay on a range where sheep will take salt freely during the spring and summer.

2) Ten per cent phenothiazine in salt, consumed at the rate of 1.5 Gm. of phenothiazine per day for each ewe and her lamb (or 0.8 Gm. per head for ewes and lambs), will not only prevent clinical nematode parasitism in the lambs, but will reduce worm numbers to a minimum in the ewes.

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Hormonal Stimulation of Estrus and Ovulation in Sheep and Goats

(A Review)

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FEMALE SHEEP and goats are sexually quiescent or partially quiescent during several months of the year. In many instances, there would be an economic advantage in having part or all of the ewes or does in a flock bred during the spring or summer months, instead of the usual time in the fall or early winter. The possibility of stimulating estrus and ovulation in anestrous does and ewes with hormones has received considerable attention in recent years. Results have been variable, and techniques that give dependable and satisfactory results in practice have not yet been developed. The object of this paper is to present a review of the work conducted to date, so that field workers and others interested in the application of these results in practice may be acquainted with the present status of the problem.

RESULTS OF RESEARCH

Seasonal Occurrence of Estrus in Sheep.—The seasonal nature of estrus in sheep is well established. Work by Küpfer,[26] McKenzie and Phillips,[36] McKenzie and Terrill,[37] Kelley and Shaw,[23,24] Schott, Phillips, and Spencer,[53] and Stewart and Moir,[56] has shown that most ewes begin coming in estrus during the early fall months. If not bred, they continue to exhibit estrus at approximately sixteen to seventeen day intervals until some time during the winter or early spring. Cole and Miller[14] state that the sexual season in mature ewes of the so-called mutton breeds is 100 to 150 days in length, and that it is shorter in ewe lambs, sometimes consisting of only one estrus. Hammond[18] points out that the time of onset and end of the breeding season appear to be evenly spaced on either side of the shortest day, on the average. There is some variation among breeds, some tending to exhibit estrus earlier in the fall and continuing to undergo cycles until later in the winter or spring. Some ewes in certain breeds may exhibit estrus during the spring and summer months, as was shown by Schott, Phillips, and Spencer[53] for Corriedale, Dorset, and Tasmanian Merino ewes. Some writers, including Marshall,[38] have stated that certain breeds, such as the Merino, may be bred at any season of the year. Küpfer,[26] Quinlan, and Mare,[48] Roux,[51] Quinlan, Steyn, and De Vos,[49] and Kelley and Shaw[24] have also studied this problem and the evidence indicates that, like other breeds, Merino ewes generally have a quiescent or anestrous season during the spring and summer months, although under certain conditions some ewes may show estrus during these seasons.

Loginova[31] observed spontaneous ovulation in fat-tailed Merino and Karakul ewes during the summer months, but it was not accompanied by estrus. Forced mating during periods of silent estrus resulted in conceptions in 6 per cent of the cases.

Pomanskii and Stojanovskaja[46] studied 500 Karakul ewes 3 to 6 years old in Uzbekistan (in U.S.S.R.) that were run with rams during the period May 25 to August 13. In the autumn, 78.3 per cent lambed. They concluded that under Uzbekistan conditions, Karakul sheep may breed all the year round. These results are in contrast with those of Schott, Phillips, and Spencer,[53] who observed no occurrence of estrus in Karakul and Karakul grade sheep from three days after each ewe lambed in the spring until middle or late August, under

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conditions at Beltsville Research Center, Beltsville, Md.

Recent observations on this Karakul flock at the Beltsville Research Center confirm earlier observations by Schott, Phillips, and Spencer.[53] The Karakul and grade Karakul ewes were last bred for regular seasonal lambing, beginning in August of 1941. After July 1, 1942, ewes were maintained with rams at all times so that mating could take place whenever estrus occurred. The lambing results through two years (from Oct. 1, 1942, to Sept. 30, 1944) indicate the seasonal nature of the breeding, and are summarized in table 1.

TABLE 1—Lambing Results of Karakul Flock at Beltsville, Md. (From Oct. 1, 1942, to Sept. 30, 1944)

YEAR	OCT.	NOV.	DEC.	JAN.	FEB.	MAR.	APR.	MAY	JUNE	JULY	AUG.	SEPT.
1942-3	0	0	15	27	24	13	20	3	3	2	5	0
1943-4	1	0	11	26	26	27	3	2	8	5	1	0
Two years	1	0	26	53	50	40	23	5	11	7	6	0
Percentage	0.4	0	11.7	23.9	22.5	18.0	10.4	2.2	5.0	3.2	2.7	0
Month of breeding	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.

There were 222 lambings. Only 21 of these represented second conceptions from breedings following rather closely after the end of a pregnancy, and all these were cases in which the ewes were not nursing lambs, the lambs having been sacrificed for pelting within a few days after birth.

Seasonal Occurrence of Estrus in Goats.—The situation in goats is very similar to that in sheep, except that after the first estrus of the season, ensuing estrous cycles are about twenty-one days in length, on the average. Küpfer[26] studied the ovaries of Swiss goats and found that ovulation definitely occurred during the months of October, November, and December, and he states that the breeding season may extend into adjacent months under certain circumstances, such as domestication. Similar observations were made on Boer and Angora goats in South Africa, except that the breeding season was approximately from April to August, owing to the reverse seasons as compared to the Northern Hemisphere.

Jeffery[20] studied 500 registrations of animals during 1933 in the British herd book and found that 2.7 per cent were born in January; 89.0 per cent between February and May; 4.6 per cent in June; and 3.7 per cent during the remaining months.

Phillips, Simmons, and Schott[44] studied Toggenburg and Saanen does and found that most does came in estrus regularly between about the middle of September and the middle of December. A few does came in estrus during July, August, and early September, and some continued to come in estrus through February and March. An occasional doe exhibited estrus during April, May, and June. Thus, the season during which most does can be bred with reasonable certainty is limited to about three months in the fall. A study of registration records by these workers revealed that, in practice, there is little difference in the seasonal distribution of births in the Nubian, Saanen, and Toggenburg breeds, and that most does kidded as the result of breedings in August to December, with most breedings occurring in September, October, and November.

For a complete review of literature on the mating behavior of female mammals and the factors conditioning that behavior, readers are referred to Young.[60]

Effects of Gonadotropic Hormones on Anestrous Ewes.—For brevity, the following abbreviations are used in this and later sections:

ap—anterior pituitary extract
fsh—follicle stimulating hormone
lh—luteinizing hormone
pms—pregnant mare serum
pu—human pregnancy urine
I. U.—International Unit
m.u.—mouse unit
r. u.—rat unit

Many attempts have been made to stimulate estrus and ovulation in anestrous ewes,

and the results are summarized briefly below. When considering this problem, it must be recognized that the hormone units used by different authors are not always comparable, and that in some cases no units are actually determined, for example, when raw plasma from pregnant mares is used. Also, there may be some confusion owing to varying definitions of the same term. Prolan, for example, has been used to designate a follicle-stimulating or a luteinizing fraction or a combination of both, and in many cases the nature of the product used is not clear.

Cole and Miller[12,13,14] found that pms would induce estrus and ovulation in the ewe. Doses of approximately 100 r.u. spaced at intervals of seventeen days were necessary to produce these phenomena. Single injections were as effective as 3 injections on consecutive days. Daily doses for seventeen days were not as effective as 2 doses at an interval of seventeen days. Estrus was also induced by 2 injections at an interval of seven days, but whether ovulation occurred in these cases was not determined. Cole[10] reports that ovulation occurs about seventy-two hours after injection of pms. In a later paper, Cole[11] states that in further experiments results ranged from practically complete failure to even better results than were obtained in initial work, and that work designed to explain the erratic results had been done without success. Cole[11] concluded that, although induction of estrus in ewes with gonadotropic hormone has some promise, a great deal of work is still necessary before such a procedure becomes practical.

Following Cole's original work, many attempts have been made to stimulate estrus and ovulation with pms. Results have been quite variable.

Boicenko,[7] in a preliminary report, states that 245 Merino x Tigai ewes were injected with 100 to 1000 m.u. of pms, beginning on May 15. The ewes began coming in estrus on June 1 and 200 were served within five days. Doses below 400 m.u. were ineffective. Similar experiments were conducted on other flocks, totaling 10,000 head, with over 80 per cent successes. It is not clear whether check lots of ewes were used to determine if any matings would have occurred normally at this season. Komjakovic and Sasenok[25] injected 2 doses of 5 to 7 cc. of pms in 410 ewes at an interval of sixteen days. In two to six days after the second injection all the ewes exhibited estrus. In another trial with 830 young ewes that had not been bred, 80 per cent showed estrus within two to six days after the second injection.

McKenzie and Terrill[37] treated 11 anestrous ewes with 300 to 400 r.u. of pms. Only 1 ewe came in estrus, and ovulation occurred in 5 animals.

Litovcenko and Litovcenko[30] injected pms into anestrous ewes during May. Three groups of 5 each received the following amounts: 200 m.u., 800 m.u. in 2 injections at sixteen-day intervals, and 2,000 m.u. in 5 injections at intervals of ten days. All ewes were killed three to four days after the last injection. Four ewes had ovulated in the first group and 3 in the second, while all ovaries in the third group showed cystic degeneration. It is not clear whether these ewes came into estrus, but it is stated that large doses induce estrus but are not recommended owing to the cystic degeneration that occurs.

O'Neal[41] reports on the field use of pms (Gonadin) on anestrous ewes designated as "Rambouillet quarter-bred Lincolns." Of 430 receiving a single injection of 250 r.u. in early May, 418 or 97.2 per cent lambed before January 31. Some of these ewes showed signs of estrus, but the rams showed no interest in breeding, so breeding was delayed somewhat until rams were stimulated by hormone treatment. Of 404 ewes receiving 2 injections at sixteen-day intervals during May, 304 or 75.2 per cent lambed, and of 296 control ewes, 77 or 26 per cent lambed by the end of January. This latter figure would indicate that not all the ewes were in anestrus at the time this work was done. Percentage lamb crops in the three groups were 104.2, 78.0, and 93.0, respectively. The control group started lambing as early as the injected groups, and the chief advantage of the treatments appeared to be in the group receiving 1

injection of pms, where the ewes tended to lamb more as a group during the early part of the season and where the lamb crop was larger.

Wunder, quoted by Frank and Appleby,[16] reported that results with pms (Gonadin) in a number of large-scale attempts to induce estrus and ovulation in range sheep had been quite variable. In some instances, an exceptionally large number of lambs were born with a considerable shortening of the lambing period, but in others no effect could be traced to the hormone.

Judovic and Fomenko[22] used fresh pms on 560 Voloshian and Karakul x Voloshian ewes during June to December and obtained estrus in 40.7 per cent, while not one of the 228 control ewes came in estrus. Five cc. was considered the best dose. Four different flocks were used, and percentages of ewes showing estrus were 55.0, 39.8, 37.6, and 20.0, while percentages of conceptions were 24.6, 10.9, 7.7, and 0. Best results were obtained with ewes that had lost their lambs, then, in order, with 15-month-old ewes, lactating ewes, and barren ewes. Of those injected in these groups, the percentages lambing were 38.9, 29.2, 23.8, and 19.5, respectively.

Polovceva and Fomenko[45] injected 37 ewes with 10 cc. pms each. Approximately 40 per cent came in estrus after seventy-two hours and the remainder within four to twenty-two days. In a large-scale test with 400 ewes similar results were obtained. Most of these ewes received 2 injections at intervals of sixteen days, but some came in estrus after the first injection. Laparotomies were performed on 57 injected ewes, 30 of which had exhibited estrus and 27 of which had not. Forty per cent of the first group and 33 per cent of the second had ovulated. Of those failing to ovulate, 14 had functional corpora lutea, which was considered a factor inhibiting ovulation. A comparison was also made of 2 groups of ewes receiving 2 successive doses of 10 cc. pms, but in 1 group the second doses were given in successive 5 cc. doses on the fifteenth and sixteenth day after the first. The respective percentages of ewes showing estrus were 20.0 and 48.0, and of lambing 7.0 and 20.5.

Quin and Van Der Wath[47] treated 110 anestrous ewes with pms, the dosage varying from 25 to 1,000 m.u. Nine animals showed estrus and some additional ewes attracted the ram but did not allow service. Thirty-three animals were slaughtered and the organs examined. Ovulation had occurred in 9 cases. Forty-three animals were bred artificially forty-eight and seventy-two hours after treatment. Four became pregnant and in all 4 cases estrus had occurred within twenty-four hours after treatment.

Ten aged ewes were injected with 250 r.u. of pms (Gonadin) in early April, by Van Der Noot, Reece, and Skelley.[58] Estrus was not induced and they were reinjected sixteen days later. All the ewes had mated within ten days and 5 conceived. These workers also injected 25 yearling ewes with 250 r.u. pms in late April, and 11 of them came in estrus and mated within ten days. The remaining 14 were reinjected sixteen days after the first injection and 7 came in estrus and mated. Five of those that mated produced lambs.

Underwood and Shier[57] treated 196 ewes with 250 r. u. pms (Gonadin) in January (Southern Hemisphere) without effect. Lambing took place at the normal time (late July and August). Twenty ewes received 250 to 500 r.u. pms in 2 injections at sixteen-day intervals in late October and early November and 10 received 500 to 1,000 r.u. Five of those receiving 250 to 500 r.u. came in estrus, compared with 7 of the 10 receiving 2 doses of 500 to 1,000 r. u., and 1 among 15 controls. Only 5 of this flock of 35 ewes lambed ahead of the usual season. These workers concluded that use of pms to advance the breeding season was not yet a practicable proposition.

Frank and Appleby[16] observed ovulation in all of 13 ewes receiving pms. Some received single and others a series of injections. Single injections ranged from 250 to 500 r.u., and total amounts given in series ranged from 275 to 1,250 r.u. Six were given 2 doses each at sixteen-day intervals, with doses of 250 r.u. at the outset and 50

or 250 on the sixteenth day. Estrus occurred in only 1 case following a single or a series of injections, but occurred in 4 of 6 ewes receiving injections at sixteen-day intervals, in one to three days following the second injection. Further work was done by Frank, Schott, and Simmons[17] to study the minimum dosage of pms that would induce ovulation and the possibility of inducing both ovulation and estrus. Estrus was produced in only 6 of 48 possible cases and ovulation in 21 cases. Doses of pms varied from 32 to 630 r.u.

Gonadotropic extracts of the urine of pregnant women (pu) have also been used in attempts to stimulate estrus and ovulation in sheep. Hammond, Hammond, and Parkes[19] observed one anestrous lamb that received 500 I. U. of pu alone and ovulated without exhibiting estrus.

Prolan has been used by several workers. Strictly speaking, prolan is gonadotropin from human pregnancy urine, but sometimes is applied erroneously to gonadotropins from other sources. Whether it was derived from pregnancy urine in all cases reviewed here is not clear. Roux[51] obtained negative results in attempts to induce estrus in anestrous ewes with injections of prolan. Mauch, Mihaila, and Hoblea[39] also failed to induce estrus during the months of June, July, and August with subcutaneous injections of 150 to 240 r.u. of prolan, given in a single dose, in 3 consecutive doses, or at intervals of two, three, five, fifteen, and twenty days. Ewes receiving 275 to 300 r.u. in a single dose or on three consecutive days reacted positively in 5.0 per cent of the cases. These ewes were served but did not conceive. Subcutaneous injections of prolan combined with intramuscular injection of a folliculin preparation also failed to induce estrus. A series of experiments by Loginova[31] showed that ovulation could be induced by injections of 100 to 400 m.u. of prolan during anestrus. Doses of 500 to 600 m.u. caused maturation of multiple hypertrophied follicles. The doses given did not cause premature luteinization. Forced mating twenty-four to forty-eight hours after prolan treatment was not followed by conception.

Extracts of the anterior pituitary gland have also been used in attempts to stimulate estrus and ovulation. Parkes and Hammond[42] state that 50 mg. of horse ap produce ovulation but not estrus in ewes, probably because follicular development is very rapid in the absence of corpora lutea, but that a second injection in fifteen to seventeen days produces ovulation and estrus. Hammond, Hammond, and Parkes[19] induced ovulation in anestrous sheep with horse ap. Some animals were given 3 daily injections and the lowest total dosage of 15 mg. produced ovulation. Other tests with dosages of 30 to 60 mg. showed that 1 injection was sufficient to produce ovulation. Ovulation was produced in 20 of the 23 animals used in these tests. The ovaries were examined, in most cases, within five days after injections were begun. A clear cut estrus was observed in only 1 of these ewes. Progesterone and pu were given in conjunction with the ap in some cases, but the effects were not clear.

Other workers have also used various combinations of gonadotropic hormones, or report on the use of various hormones on different ewes in the same experimental flock. Estrogens were used in combination with gonadotropins in some cases and are included here. Zavadovskii[61] treated over 2,000 anestrous sheep with prolan, ap, and whole pu. Ovulation was induced in 90.0 per cent of the cases examined, and occurred eleven to forty-eight hours after injection. During the normal breeding season, ovulation was induced by injections during the estrous cycle, but in the majority of the cases estrus did not occur and the normal cycle remained unchanged. Six of eleven ewes bred twenty-four to thirty-six hours after injection in midcycle became pregnant.

Zavadovskii *et al.*[72] injected 200 to 2,000 m.u. of prolan or 1,500 m.u. of folliculin followed by prolan into anestrous animals and obtained varying grades of ovarian stimulation and production of corpora lutea in over 80.0 per cent of the 57 animals. None of the ewes showed signs of estrus. Fresh corpora lutea were also induced in nine milking sheep with 400 to 800 m.u. of

prolan. Results were confirmed in field trials with over 1,000 ewes, using prolan alone or with folliculin, 10 to 100 cc. of pregnancy urine or 0.25 to 1 Gm. of powdered pituitary. None of the animals showed estrus but all those examined had fresh corpora lutea. Some ewes were force-mated after treatment. Of 29 bred four to five days after treatment, none conceived, but it was found that ovulation occurred twenty-four to thirty-six hours after injection, so force-mating was done at the time of injection or within one to three days in 70 ewes and 11 produced lambs. Also 250 were artificially inseminated and only 27 lambed.

Bell *et al*,[8] regularly produced ovulation without estrus by injections of pms (100 r.u.) or sheep ap (2.5 Gm. equivalents). Combinations of pms and estradiol benzoate (Progynon-B) were generally unsuccessful in producing both estrus and ovulation. These results are in agreement with those of Cole and Miller,[14] who report that combinations of pms and estrogens are usually not successful in producing both estrus and ovulation.

Hammond, Hammond, and Parkes[19] injected 25 anestrous ewes with pms, the dose varying from 100 to 2,000 I.U., and all ovulated. Eleven animals received pms alone and none came in estrus. Two of 3 animals that received 2 doses each at fifteen-, sixteen-, and seventeen-day intervals came in estrus at twenty and twenty-one days after the first injection. Three animals received a supplemental treatment of pu but none came in estrus. Six animals received a supplemental dose of estradiol or stilbestrol and 4 came in estrus. In addition to the above, 2 ewes were given stilbestrol by mouth and in the vagina but neither estrus nor ovulation resulted. Progesterone (2.0 mg.) was given to 1 lamb and a doubtful estrus, without ovulation, resulted.

Several thousand anestrous ewes that were maintained on natural pasture in the Ukraine, Crimea, and North Caucasus were injected with prolan, pu, pms, prolan + milk lysates, or prolan + menopause prolan. Zavadovskii, Paduceva, and Wunder[70] report that the overwhelming majority of ewes ovulated within seventy hours, but only weak symptoms of estrus were observed with almost no willingness to accept the ram. Some individuals in good bodily condition showed all signs of estrus. Prolan, in doses of 250 to 500 m.u., had a quicker follicle-stimulating effect than pms at the same dosage; ovulation occurred in twenty-four to thirty and forty to fifty-four hours, respectively. Forced mating resulted in 0 to 53 per cent lambings in the various groups, the highest percentage occurring in ewes receiving 250 to 500 m.u. of prolan and being inseminated twenty-four to thirty-six hours thereafter. Low percentage of pregnancies was attributed to physiological unreadiness of the genital tract to receive the zygote, and individual variations in occurrence of estrus to bodily and nutritional conditions of the ewes.

Effects of Gonadotropic Hormones on Ewes at Beginning and During the Natural Breeding Season.—In addition to attempts to stimulate estrus and ovulation in anestrous ewes, considerable work has been done with ewes at the beginning of and during the regular breeding season. This work has included attempts to increase the number of multiple births, to shorten the breeding season, and to treat sterility.

McKenzie and Bogart[35] treated ewes with pms, given at sixteen-day intervals, thus obtaining 70.0 per cent of ewes showing estrus during a given period. In the controls, 35.0 per cent came in estrus during the same period. Two doses of 250 to 500 r.u. were most effective.

Bludorov and Dubrova[5] treated 107 ewes that failed to come in estrus during the normal breeding season, with gravidan (probably pu). Autopsy showed that ovulation took place on the first or second day following injection, but the induced ovulation was not accompanied by estrus, except in 30.0 per cent of the ewes that accepted the ram. The others were artificially inseminated. In a later report (Bludorov and Dubrova[6]) it is stated that 56.0 per cent of the ewes in which ovulation was induced produced lambs.

The fat-tailed Tabasar breed of the north-

ern Caucasus has a normal breeding season beginning in the second half of June. Litovcenko,[20] using adult and young (fourteen to fifteen months old) ewes, began injections of pms on June 1. One to 3 injections of 2 to 11 cc. were given at intervals of sixteen days. Sixty per cent of treated mature animals and 55.5 per cent of the young ewes came in estrus. In the controls, 23.0 per cent came in estrus. Controls began coming in estrus ten to seventeen days later than injected ewes. Doses of 2 to 4 cc. were most effective, and best results were obtained with 2 doses in mature and 3 in young ewes. Similar experiments, carried out during December and January, gave the same results. At this time, the majority of both control and experimental animals came in estrus in the equivalent of two to three cycles after lambing.

Judovic[21] reports that Voloshian ewes normally breed from August to February and Voloshian x Merino ewes from July to March. Injections of 40 to 400 m.u. of pms into crossbred ewes between July 18 and August 8 induced estrus in 16 out of 17 ewes or 94.0 per cent. Among the controls, 25.6 per cent came in estrus. In ewes injected between August 8 and 29, incidence of estrus was not increased, when compared with controls. In all the trials, conception rate in injected ewes was 64.0 per cent, and in the control ewes it was 87.2 per cent.

Zavadovskii, Margulis, *et al.*[63] used doses of 100 to 500 m.u. of pms and had greater success with the higher doses in inducing estrus in ewes. Results were not consistent in two years. In one favorable season, 169 ewes were injected, and 54 (39.9%) came in estrus, compared with 8.5 per cent in the controls. Twenty-three were mated and 10 (43.5%) lambed. The 115 animals which did not come in estrus were inseminated and 22 (19.1%) lambed. Of 262 ewes to be injected twice, 149 came in estrus after the first injection, and of the remaining 113, 28 came in estrus following the second injection sixteen days later. The percentages of ewes lambing in the 2 groups were 43.5 and 33.3, respectively. The work was repeated the following year, after a hard winter, and none of the controls and only 1.0 per cent of the injected ewes came in estrus. Only 5.0 per cent of those inseminated thirty-six to forty-eight hours after injection with pms produced lambs, while 15.1 per cent of those receiving pms + prolan lambed.

Lopyrin, Loginova and Babicev[33] carried out large scale experiments in attempting to increase fertility in 477 Karakul sheep. A flock of 418 served as controls. Pms, from mares fifty-two to seventy-three days pregnant, was given, in 2 doses. The first was on the fourteenth day of the cycle and the second after the onset of estrus. Examination of ovaries forty to fifty hours after injection in 62 ewes showed one to seven ovulating follicles, and 3 to 10 cc. was found to be the optimal dose. The lambing rate in ewes that conceived after the first insemination was 166.8 per cent and 119.1 per cent, in the controls.

Several workers have used pu or prolan in attempts to stimulate ewes at the beginning of, or during, the breeding season. Zavadovskii *et al.*[72] injected 56 ewes, during the normal breeding season, with doses of 150 to 500 m.u. of prolan or 25 cc. of pregnancy urine, six to fourteen days after an estrous period. Ovulation was induced but only one conception resulted from forced matings or artificial insemination. The treatments did not disturb normal periodicity. These authors also observed that removal of the normal corpus luteum in 9 ewes resulted in shortening the cycle by five to eleven days, while removal of the experimental corpus luteum did not disturb normal periodicity. Zavadovskii and Paduceva[64] attempted to produce superfetation in Karakul and Merino ewes by injections of pregnancy urine (150 to 500 cc.) on the day of normal estrus, and also one to four days before and two and one-half to three days after estrus, but each ewe produced only 1 lamb. Extra ovulations resulted from postestrous injections, but forced matings did not result in additional lambs.

Lysov and Stojanovskaja[34] treated 4 groups of approximately 412 Karakul ewes with prolan. The first group received prolan thirteen to fourteen days after the preceding estrus and was inseminated once

during the subsequent estrus. The second was injected at the beginning of estrus, and all were inseminated at the same time and half a second time, twelve hours later. The third and fourth groups were inseminated once during estrus and injected with prolan and also inseminated twenty-four and forty-eight hours later, respectively. Prolan was effective in increasing the incidence of twinning in the first group only.

Lopyrin[32] treated 380 Merino x Valahian sheep with 10 to 60 cc. of pu or 100 to 500 m.u. of total prolan two to eight days before the onset of estrus. Examination of ovaries of 146 experimental and control animals revealed that the optimum time for injection was two to five days before estrus and that the optimum dose was 100 to 400 m.u. of prolan. This treatment induced synchronous polyovulation with as many as seven to eight mature follicles. Lambing records for 246 experimental ewes showed that fertility was raised by optimum dosage and timing, for 65 ewes so treated produced 120 lambs (187%) as compared with an average of about 130 per cent in controls. In about 50 to 60 per cent of the cases, injection of prolan was either ineffective or resulted in ewes not settling or caused a delay in the onset of estrus. The lambing percentage of the entire experimental group was only slightly above the controls.

Riet, Echenique, and Jaunsolo[50] injected 125 units of prolan into 4 maiden and 4 adult ewes about one week after estrus. The intervals between first and second estrus differed little from those of controls, but the interval was definitely longer in maiden ewes. The average intervals for adult and maiden experimental ewes were sixteen and forty-nine, and for the controls fifteen and a half and fifty-four and six-tenths days, respectively.

Extracts of ap have also been used on ewes during the breeding season. Warwick and Casida[58] injected 35 ewes with a fsh-containing extract of sheep pituitary twelve to fifteen days after onset of estrus, followed by an intravenous injection of lh on the seventeenth day. The duration of the next estrus was increased from one and forty-eight hundredths to two and twenty-four hundredths days, the increased length being due to ewes coming in estrus sooner. None of the 5 ewes treated three to nine days after beginning of estrus came into estrus as a result of the treatment. Murphree *et al.*[40] produced superovulation in ewes by treatments with a single injection of follicle-stimulating extract of ap on four consecutive days and an intravenous injection of 10-Gm. equivalents of luteinizing extracts of ap or 1-Gm. equivalent of unfractionated extract. All ewes were artificially inseminated on the day of the intravenous injection and again on the following day. Twenty-four ewes, upon which treatments began twelve days after the preceding estrus, had a total of 576 corpora lutea and yielded 357 eggs, of which 153 were fertilized. Five ewes upon which treatment began three days after beginning of estrus had 57 corpora lutea, yielded 25 eggs and none were fertilized. Nine anestrous ewes, treated with follicle-stimulating extracts, had 84 corpora lutea and 25 unfertilized eggs. Four anestrous ewes treated with pms or a combination of pms and unfractionated ap had eight corpora lutea and eight eggs, and one egg was fertilized. Casida, Warwick, and Meyer,[9] using follicle-stimulating and luteinizing-hormone treatments similar to those used by Murphree *et al.*[40] began treatments twelve days after the preceding estrus, produced superovulation, and a high proportion of the ova were fertilized. Rate of survival of the fertilized ova was quite low.

Casida, Dutt, and Meyer[8] treated 30 ewes with either an unfractionated ap or a follicle-stimulating ap extract. Ewes were treated at the fourth, eighth, or twelfth day of the cycle, and received 4 subcutaneous injections of either of the above extracts, followed by one intravenous injection of unfractionated extract. Unfractionated extract increased both the length and variation of the estrous cycle in which treatment began. The follicle-stimulating extract had no evident effect, except when given on the eighth day, when an increase in variability resulted. Treatment with either extract shortened the subsequent estrous period, except when follicle-stimu-

lating extract was given at twelve days, in which case the subsequent estrus was longer than in controls. Neither extract gave any practical gain in controlling time of estrus so that variation in the time of breeding could be decreased.

Other workers have used various combinations of hormones in the treatment of ewes, or have treated comparable groups of ewes with various hormones.

Litovcenko[28] worked with North Caucasion Merino ewes from July 1 to August 8. Thirty-six control ewes began coming in estrus during the last days of this period. Twenty-nine ewes received 1 to 3 injections of prolan, with dosages varying from 20 to 40 to 200 to 300 m.u.; 21 (72.0%) came in estrus and 19 (65.5%) produced lambs. Best results were obtained with 100 to 200 m.u. given in 2 doses. Twenty-nine ewes received 20 to 100 cc. pms from 2 pregnant mares; 25 came in estrus and 17 produced lambs. The lowest dose gave the best results.

Steffens[54] treated a flock of Karakul ewes, 63 of which had failed to come in estrus, with various hormones. One group received prolan and 23.0 per cent came in estrus within eight days. Of 52 ewes given "yohimbine-veratrine," 27.5 per cent showed estrus within the same period. "Provetan" (Progynon-B) induced estrus in 94.0 per cent of the cases, and the optimum dose was reported to be 200,000 i.u. Sixteen animals received 100,000 m.u. of "unden" (an estrogenic hormone) and 81.2 per cent came in estrus. The exact time of this work, in relation to the normal breeding season, is not clear.

Zavadovskii, Paduceva, *et al.*[68] treated ewes with various gonadotropins (prolan, menopause prolan, pms, pu prolan, and menopause prolan + pu prolan) and attempted to recover ova from the fallopian tubes. The highest percentage of ova was recovered from control ewes. Only 13 ova, 9.4 per cent of the number of corpora lutea, were recovered from treated animals, while 61.0 per cent recovery was obtained in 31 control ewes in natural estrus. In further work, Zavadovskii, Paduceva, *et al.*[63] found that 18 of 36 ova recovered after normal estrus were in different stages of segmentation, and 11 out of 20 from ewes in which the normal cycle corpora lutea had been previously removed were in the same state of differentiation. Of 42 ova recovered after stimulation of ovulation with gonadotropins, none were fertilized. Further work by Zavadovskii[62] with ewes during July, injected with prolan and pms, gave similar results. Most of the ewes ovulated within twenty-four to seventy-two hours but only 2.5 per cent showed clinical signs of estrus and willingness to accept the ram. After single injections of gonadotropin, about 81.0 per cent (compared with 46.0 per cent in controls) ovulated and showed signs of estrus fifteen to sixteen days after the injection. In ewes receiving a second injection on the sixteenth day, a great number ovulated, and 15.0 to 20.0 per cent came in estrus within seventy-two hours, exhibiting a heightened sex drive for about a fortnight.

Zavadovskii and Paduceva[65] treated 8 groups of Tigai ewes as follows: (1) 29 ewes with pms on first day of estrus; (2) 28 ewes with pu on first day of estrus; (3) 80 ewes with pms on day after estrus; (4) 23 ewes with pu on day after estrus; (5) 28 ewes with pms on sixth day after estrus; (6) 10 ewes with pu on sixth day after estrus; (7) 48 ewes with pms thirteen to fifteen days after beginning of last estrus; and (8) 43 ewes with pu thirteen to fifteen days after beginning of last estrus. The average percentages of lambings in the respective groups were 140, 147, 129, 119, 128, 157, 170, and 140, compared with 128 per cent in 504 controls. The percentages of conceptions after the first insemination were 65 in the experimental animals and 81 in the controls.

Zavadovskii and Paduceva[66] used pu and prolan to stimulate ovulation. The percentage of twinning was increased, but there was also a slight increase in barrenness, and the total lamb crop increased slightly. Pms gave slightly better results than the other substances, increasing the lamb crop by 26.0 per cent in adult ewes and 18.0 per cent in yearlings. Ovaries in 17 injected ewes were examined and found

to have 2.23 corpora lutea per ewe, compared with 1.54 in controls.

Hammond, Hammond, and Parkes[19] treated ewes with horse ap (50 mg.), ap (50 mg.) and pu (50-250 I.U.) combined with pms (400 to 2,000 I.U.) at various stages of the estrous cycle during the normal breeding season. They concluded that when treatments were given while a corpus luteum was active, both ovulation and estrus were usually suppressed. If the corpus luteum were regressing, the estrus occurred at about the time it would have in a normal cycle. When ap was given a few days before the corpus luteum was due to regress, superovulation occurred at the next estrus.

Effects of Gonadotropic Hormones on Anestrous Does.—Stevenson[55] injected 13 anestrous does with 200 r.u. of pms (Gonadin) each, and repeated the dose in sixteen days. Three to five days following the second injection, there was marked evidence of estrus in all does. They were bred and 55.0 per cent conceived.

Sandburg[52] has reported some success in stimulating extraseasonal breeding in goats by injection of pms (Gonadin).

Phillips, Simmons, and Schott[44] report results of preliminary work with goats. In attempts to induce extraseasonal breeding, 20 does that had been dry during the preceding winter were treated with pms. Nineteen exhibited estrus and were bred and 6 produced kids. Five lactating does were treated with pms. Four came in estrus and 1 conceived. The results indicated that 400 r.u. of pms were sufficient to induce estrus in most does. However, further work on does in the same flock was conducted the following year by Frank, Schott, and Simmons[17] with somewhat different results. In a total of 97 cases in which anestrous does received 150 to 400 r.u. pms in from 1 to 3 doses, only 7 exhibited estrus. Operations were performed in 28 cases, and in 26, ovulation had occurred.

Effects of Estrogenic Hormones on Anestrous Ewes.—Cole and Miller[14] treated anestrous ewes with estrogenic hormone (estrin). Estrus was not produced with any regularity with doses less than 3,000 r.u., although 2 ewes responded to doses of 400 r.u. and 800 r.u., respectively. When estrin was given simultaneously with pms, only 2 of 10 ewes ovulated, and in only 1 ewe were ovulation and estrus observed, and it was concluded that estrin inhibited ovulation. When estrin was given two days after pms, ovulation was not inhibited, but estrus was not induced.

McKenzie and Terrill[37] produced estrus in anestrous ewes with 520 r.u. of estradiol benzoate (Progynon-B) but the most regular response was obtained with 900 to 1,500 r.u. Fifty-two cases received single injections of 800 r.u. and estrus occurred in 44.0 per cent of them, in from twenty-one to 174 hours with an average of fifty hours. During the normal breeding season, injections of estradiol benzoate were effective in producing estrus only when given near the time of normal estrus.

Anderson[1] injected estradiol benzoate into anestrous lactating, high-grade Merino ewes and obtained estrus in 74.3 per cent of them within twenty-one days, usually one to five days after injection or about seventeen days later. The maximum response was obtained with 5,000 to 6,000 I.U. The induced estrus was often followed by others at regular intervals. Estrus, whether induced or occurring subsequently, was not always accompanied by ovulation. Ovulation without estrus was observed in untreated ewes during the anestrous period.

August[2] produced estrus in anestrous ewes with 10,000 and 15,000 I.U. of estradiol benzoate. Of 4,281 animals treated, 72.5 per cent mated and 43.1 per cent lambed. Estrus appeared three to seven days after hormone injection.

Bell *et al.*[3] produced estrus in 5 out of 8 ewes with estradiol benzoate (Progynon-B) but none of the ewes ovulated.

Bell, Casida, and Darlow[4] induced estrus in 21 out of 22 spayed yearling ewes by injections of 1,000 r.u. of estradiol benzoate. Nine came in estrus on the day following injection and the remainder on the second day. The average duration of estrus was two and eight tenths days. Progesterone, given in 8 successive daily doses (20 to 64 guinea-pig units) beginning on the fourth day after treatment with es-

tradiol benzoate, increased the duration of estrus to three and four tenths, three, and four and a half days in three groups of ewes.

Hammond, Hammond, and Parkes[19] treated 15 anestrous ewes with 0.25 to 2.0 mg. of stilbestrol or estradiol. Three received 0.5 to 1.0 mg. of estradiol and estrus and ovulation were observed in 2 by the end of five days and in 1 at the end of three days, although in this case the estrus was doubtful. In 2 cases receiving stilbestrol, animals were sacrificed at one and two days, and neither estrus nor ovulation had occurred. Ten animals receiving stilbestrol were slaughtered three days or more after treatment. Six exhibited estrus and 6 ovulated, but estrus and ovulation occurred together only in 3 of the cases, and the only case that exhibited neither estrus nor ovulation received only 0.25 mg. stilbestrol.

Frank and Appleby[16] induced estrus in 11 of 12 anestrous ewes by injections of 2 mg. stilbestrol on the first and one mg. on the second day. When given in conjunction with pms, stilbestrol appeared to inhibit the action of the gonadotropic hormone, since ovulation occurred in only 7 of 15 cases, as contrasted with ovulation in all of 13 cases where pms was used alone. They concluded that stilbestrol, either alone or in combination with pms, did not appear to provide the hormonal stimulation necessary for successful fecundation in anestrous ewes.

Quin and Van Der Wath[47] treated 43 anestrous ewes with 1 to 5 mg. of stilbestrol, intramuscularly. Eight animals came in estrus and 8 others showed doubtful signs of estrus. Laparotomies were performed on certain of these 43 ewes and ovulation had occurred in most cases, whether the ewes had exhibited estrus or not. Seven ewes were injected with 1 mg. of stilbestrol during the breeding season, on the eighth day after a normally occurring estrus, in each case. One animal came into full estrus within twenty-four hours. This animal and 2 others that did not show estrus were laparotomized within forty-eight hours and all had ovulated. Five spayed ewes were treated with stilbestrol on three different occasions, twelve, sixteen, and seventeen months after spaying. With doses of 5 or 1 mg., all showed estrus within forty-eight hours and allowed service for two to three days. With a dose of 0.5 mg., 2 came into estrus and 3 remained negative. Quin and Van Der Wath concluded that seasonal anestrus in ewes is closely associated with a decreased physiological response of the uterus and vagina to estrogen.

In several of the papers reviewed previously, ovulations are reported following treatments with estrogens and with synthetic products, estradiol benzoate and stilbestrol. It is probable that some, if not all, of the ovulations following treatments with naturally occurring estrogens did not result from the injections, but had occurred during "silent estrus" during the nonbreeding season. However, there is some indication that stilbestrol, and possibly other estrogens, may induce ovulation, apparently indirectly by stimulating production or release of gonadotropins from the pituitary (Hammond, Hammond, and Parkes,[19] Quin and Van Der Wath[47]).

Effects of Hormones on Males.—Rams and bucks produce spermatozoa throughout the year, but the quality of semen tends to be low during the summer months (Phillips *et al.*[43]). Libido also appears to be reduced during the spring and summer months. These two factors may be of considerable importance in obtaining fertile matings during estrous periods stimulated outside the usual breeding season. Kuznetzova and Kuznetzov[27] report beneficial results from injection of prolan into teaser rams and state that 3 or 4 injections of 1 to 2 cc. were sufficient to restore energy and vitality throughout the mating season. O'Neal[41] claims beneficial results from injections of rams with pms (Gonadin).

CONCLUSIONS

The results of the many attempts to induce estrus and ovulation in anestrous sheep and goats by the use of gonadotropic hormones may be summarized as follows:

1) Follicular growth and ovulation can

be induced in a high percentage of animals injected with gonadotropins.

2) Induction of estrus in conjunction with ovulation, as a result of treatments with gonadotropins, has been quite erratic, and no satisfactory explanation of the variable results has been found.

3) Fertility, as measured by percentage of conceptions, is generally lower in animals in which estrus has been induced or in those force-mated after induction of ovulation, than in animals bred during natural estrus. However, satisfactory fertility has been reported in some cases.

Estrogens, used either alone or with gonadotropins, have given variable results. Results of trials with gonadotropins and/or estrogens during the natural breeding season have also been quite variable. In some cases, apparent increases in fertility, as judged by percentage of conceptions or lambing percentages, have resulted. In other cases, no increase or an actual lowering of fertility has resulted.

The variable results are difficult to explain. The identity of hormone preparations can never be assured in the absence of chemical purity, and no protein gonadotropin is now marketed in this form. It does not appear probable that this accounts in any large measure for the inconstancy of estrus response in ewes and does, owing to the relatively uniform response of laboratory animals to these same gonadotropes.

Constitutional and environmental factors may play a considerable part in determining the response to specific hormone treatments. These factors, if they do play an important part, are easily overcome in the induction of follicular growth and ovulation. The extent to which they influence expression of estrus is not clear, and cannot be fully understood until the mechanism of induction of estrus is determined in ewes and does. A working basis for approaching this problem is furnished by Dempsey, Hertz, and Young,[15] who advance the hypothesis that after the animal has been conditioned with estrogens of ovarian origin, estrus is brought about by the immediate action of progesterone which is elaborated in the preovulatory follicles shortly before ovulation, as a result of release of luteinizing hormone by the anterior pituitary.

The occurrence of estrus after 2 injections of pms, at an interval approximating the normal cycle, indicates that the corpus luteum induced by the first injection of pms may have a regulatory rôle. Also it has been observed (McKenzie and Terrill[37]) that most ewes ovulate at least once before the first estrous period of a natural breeding season. Hammond, Hammond, and Parkes[19] state that horse ap and pms induce estrus only in animals having a regressing corpus luteum; that in the absence of a corpus luteum ovulation occurs without estrus, and that in the presence of an active corpus luteum both ovulation and estrus are usually suppressed. Work by Zavadovskii, Paduceva, and Wunder[69,71] indicates that corpora lutea, induced by injection of prolan or pms, are not in all cases similar to spontaneous cyclic ones as regards function and capacity to regulate the sexual cycle.

From work with laboratory animals, and the results with sheep and goats, it seems certain that time sequences in hormonal action are involved critically in the expression of normal and induced estrus. There is obvious need for further work to determine (1) The endocrine physiology of normal and experimentally induced estrus in sheep and goats and (2) the specific doses and time sequences of hormones that will induce estrus and ovulation in anestrous ewes and does.

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A Study of Bovine Infectious Keratitis

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BOVINE INFECTIOUS keratitis is a disease affecting cattle of all ages and breeds, characterized by an acute inflammation of the eyeball and its surrounding tissues. It is sometimes referred to as "pinkeye."

Although having a low mortality, infectious keratitis has long been recognized as a specific infectious disease of considerable economic importance. Dairy cows affected with the disease may drop 25 per cent or more in milk production and become unthrifty. Beef cattle, particularly young stock on which the owner is depending for a rapid gain in weight, may fail to gain, or even lose weight. Western range cattle are subjected to many hazards when their sight is impaired. For example, drowning, falling from high places, and inability to obtain proper food are not unusual on the western ranges.

Specific ocular infections which resemble infectious keratitis clinically have been reported from Canada, The British Isles, Germany, France, South America, and South Africa.

In spite of the obvious importance of this disease, very little is known of its cause and the means by which it is transmitted from animal to animal. For these reasons, the measures employed in its treatment and control seem inadequate.

Since control and treatment of infectious diseases are facilitated by knowledge of the causative agent, and there seems to be a definite need for more adequate means of controlling this disease, an attempt was made to determine its etiology.

SYMPTOMATOLOGY AND PATHOLOGY

The symptoms and lesions of infectious keratitis can best be described under two headings: acute ophthalmitis and ulcerative keratitis.

Acute Ophthalmitis.—Infection may occur in one or both eyes, separately or simultaneously. The severity varies considerably.

Acute ophthalmitis presents the symptoms and lesions seen in the first stages. The first symptoms usually observed are an acute conjunctivitis, blepharitis and a lacrimation so copious that the area below the eye is kept constantly wet. Flies are attracted in great numbers by this discharge. The iris partially or completely occludes the pupil and may appear to be distended forward. The distension is probably due to increased pressure in the posterior chamber and vitreous body. The iris may be obscured by the opaque cornea.

Varying degrees of corneal opacity appear on the second to the fourth day. During this time, the appearance of the cornea may vary from a slight cloudiness to a complete opacity. In the latter case, the circumcorneal vessels will often start to encroach into the corneal tissues and impart a bright red periphery to the opaque cornea. Due to the increased intraocular pressure, the cornea will sometimes appear markedly distended, particularly when the symptoms are pronounced.

Close observation on the third or fourth day will usually reveal a very small, centrally located corneal ulcer. A small pimple-like protuberance sometimes precedes the ulceration. The depth of ulcers varies with the severity of the infection. If only the two outer cellular layers (epithelium and Bowman's membrane) are ulcerated, the cornea heals completely in one or two weeks. If the ulceration penetrates the substantia propria to any extent, the disease may progress to the chronic type. As the disease progresses, the discharge tends to become more mucoid.

Due to pain and photophobia, affected cattle stand in the shade with their eyes

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partially or completely closed and their heads lowered. Vision is impaired in degrees varying with the corneal opacity.

Chronic Ulcerative Keratitis.—A common sequel to acute ophthalmitis, especially when animals are not properly treated, is a chronic disease complicated by secondary pyogenic bacteria. The cornea becomes extensively and deeply ulcerated and thickened by an increase of interstitial tissue in the substantia propria. It becomes dull grey and a yellow, purulent, fibrinous deposit is often evident in the ulcerated area. The circumcorneal vessels are visible in the cornea. The discharges become thicker and more purulent. Frequently, Descemet's membrane and its endothelium prolapse through the ulcerated substantia propria. If these membranes rupture, the infection readily enters the anterior chamber and produces a hypopyon.

Wind, dust, bright sunlight, flies, and poor nutrition are generally thought to aggravate the infection. Recovery depends to a great extent upon the amount of tissue destroyed and the care the animal receives.

Infectious keratitis is a seasonal disease occurring enzoötically during the summer and early autumn, but sporadic cases may occur throughout the year. It occurs in practically every part of the United States.

The morbidity in an affected herd varies, considerably, with environmental conditions. Usually, from 50 to 60 per cent of all animals in a herd become infected no matter what treatment or control measures are employed.

The most common source of original outbreaks is the introduction of infected cattle. Once established on a premise, the disease may occur every year and spread to other farms.

The incidence is higher in animals under 2 years of age than in older animals, particularly where the disease occurs every year.

REVIEW OF THE LITERATURE

Most of the literature on infectious keratitis deals with the symptoms, treatment, and control. There are remarkably few reports on its etiology.

Allen[1] reported the finding of a gram-negative diplobacillus in the ocular discharges of infected cattle, and briefly described the organism. He reproduced the disease in 1 calf by introducing the eye discharges of several infected cattle into its eyes. One attempt was made to determine the infectivity of the diplobacillus for cattle by injecting a pure culture of that organism into the conjunctival sac of 1 calf. The calf did not react to the inoculation.

Little and Jones[2] investigated an outbreak of a cattle disease which they termed infectious ophthalmia and recovered a gram-negative diplobacillus from the eyes of 24 infected cattle in the one herd investigated. The symptoms described were similar to those of infectious keratitis. Morphologically, the organism closely resembled the one reported by Allen. A complete description of the morphological and cultural characteristics was reported. Little and Jones reproduced the disease by instilling the eyes of healthy cattle with ocular discharges from naturally infected cattle, or by using pure cultures of the gram-negative diplobacillus.

Bacteriological examinations of the eyes of 10 naturally infected cattle which had completely recovered four months previously revealed the organism in 5 animals.

Flies were suspected as the disseminating agent by Little and Jones[3], but their experimental work did not confirm this supposition. They found that the gram-negative diplobacillus lived only three hours on the outside of the fly's body, and that the organism was rapidly destroyed in the digestive tract of flies.

Hauduroy[4] later classified the organism isolated by Little and Jones as belonging to the genus Hemophilus and named it *Hemophilus bovis*.

Farley[5] infected cattle experimentally by inoculating virulent eye secretions of active and recovered field cases into the eyes of susceptible cattle. Occasionally he found cattle that resisted infection. Experimentally induced infections did not progress to chronic ulcerative keratitis. Farley suggests that the failure to progress to the chronic form might have been due to the ideal conditions under which the experimental animals were kept.

According to his results, the animals developing only a mild infection could be reinfected, but those which suffered from the acute or chronic form were not susceptible to subsequent exposure.

The lacrimal secretions of animals which had recovered from the acute form of infectious keratitis did not remain virulent longer than three months.

Bacteriological examinations of the eyes of naturally infected cattle revealed, *Escherichia coli*, *Corynebacterium pyogenes*, streptococci, staphylococci, and *Pasteurella boviseptica* to be present. None of these organisms repro-

duced the disease when inoculated into the eyes of healthy calves.

Farley collected virulent eye secretions from numerous cattle during three years of study, and bacteria-free filtrates were used to inoculate the eyes of 42 susceptible cattle. No reaction was obtained in any animal. Approximately 70 per cent of the animals inoculated with the filtrate proved to be susceptible to exposure with known virulent eye secretions. The period of incubation in experimentally induced infections varied from three days to three weeks.

Farley attempted to transmit infectious keratitis from cattle to sheep and a specific eye disease of sheep to cattle, but failed in both instances.

EXPERIMENTAL PROCEDURES AND RESULTS

Investigational work on infectious keratitis was conducted for two years. Etiological studies were given primary consideration.

The combined works of Allen[1], Little and Jones[2, 3], and Farley[5] indicated that a bacterium and not a filterable agent was the cause of infectious keratitis. For this reason the investigation undertaken was carried out along that line.

Epizoölogy.—Thirteen infected herds of cattle in the central area of Ohio were investigated. The infection was found to occur in from 50 to 90 per cent of the cattle in these herds.

The most common source of a new outbreak of infectious keratitis was from the introduction of infected cattle, particularly the feeder type shipped in from the western states. In most cases, there was direct contact between the native and the infected cattle which facilitated transmission of the disease directly from animal to animal.

Four of the herds investigated gave a history of the infection having occurred every summer for several years. No infected cattle were known to be introduced into these herds. The spread of the infection to neighboring farms was common, particularly when the pastures were adjacent.

BACTERIOLOGICAL STUDIES

Specimens for bacteriological examination were obtained from the eyes of cattle by means of sterile cotton swabs. Considerable care was exercised to eliminate contamination of the swab by microörganisms such as *Bacillus subtilis,* which are commonly found on the eyelids and eyelashes.

A sample was obtained from an animal by the following technique: The head was securely restrained to eliminate accidental injury to the ocular structures; the eyelids were forced open and the swab was placed upon the conjunctiva; the eyelids were permitted to close over the swab, causing a slight irritation and resultant lacrimation; after the swab was saturated, the eyelids were forced open and the swab removed.

The tip of the swab was used to inoculate a plate of blood-agar (nutrient agar containing 4 to 5 per cent citrated horse blood). The cultures were incubated for twenty-four hours at 37 C. and then examined.

Direct smears were prepared of the exudate by expressing all the liquid possible from the cotton upon a glass slide, and permitting it to dry slowly aided by a flame. The slides were stained by a modification of Gram's method.

The direct smear of the exudate was examined microscopically to give an indication of the types of organisms that could be expected on the blood plate.

The various colonies found on the culture plates were examined, and stained preparations were made from them. No attempt was made to identify every organism found on the culture mediums but rather to determine such general characteristics as the Gram reaction, hemolytic ability, size, shape, and colony characteristics.

Studies of the Flora of Normal Bovine Eyes.—Before being able to attach any significance to the bacteria found in the eyes of cattle suffering from infectious keratitis, it was deemed necessary to determine the microörganisms in the normal eyes of cattle. Both eyes of 20 apparently normal cattle were examined bacteriologically by the methods described. The following microörganisms were present:

1) Nonhemolytic staphylococci were found in both eyes of 13 cattle and in one eye only in each of 4 cattle.

2) Hemolytic staphylococci were found in both eyes of 4 cattle and in one eye only in each of 7 cattle.

3) Nonhemolytic streptococci were found in both eyes of 3 cattle and in one eye only in each of 5 cattle.

4) Hemolytic streptococci were found in only one eye in each of 4 cattle.

5) Corynebacterium sp. were found in both

eyes of 1 animal and in one eye only in each of 5 cattle.

6) Saprophytic actinomyces were found in both eyes of 1 animal and in one eye only in each of 5 cattle.

7) *Escherichia coli* was found in one eye only in each of 2 cattle.

8) Gram-positive unidentified rods were found in one eye only in each of 6 cattle.

9) Gram-negative unidentified rods were found in one eye only in each of 3 cattle.

10) Gram-negative unidentified cocci were found in both eyes of 3 cattle and in one eye only in each of 8 cattle.

H. bovis was not found in any of the eyes of the cattle examined.

One eye of each of 2 cattle was found to be sterile. It was not unusual to find only 3 or 4 colonies on the culture plates.

Studies of Infected Eyes.—Preliminary bacteriological investigations of infected eyes made it apparent that a high percentage of them contained an organism morphologically identical to *H. bovis*. A thorough study of the cultural characteristics proved the organism to be *H. bovis*. Further investigation was confined to determining the incidence of that microörganism in infected eyes.

H. bovis was readily detectable on the direct smears of the eye discharges because of its peculiar diploid form and gram-negative staining property. Cultures did not always result in the isolation of *H. bovis* even though it was observed on the direct smear. Some failures to cultivate the organism were attributed to the presence of rapidly growing saprophytic bacteria which overgrow the other colonies on the culture. Also, cultures sometimes resulted in the isolation of *H. bovis* while it was not observed on the direct smear.

Samples were obtained from both eyes of 62 cattle in 13 infected herds in the farming area around Columbus, Ohio. One specimen from each of 22 cattle was submitted from California* and Montana.†

At the time of examination of the 62 cattle in the Columbus area, 28 (45.2%) were suffering from bilateral infections and 34 (54.8%) had unilateral infections. No attempt was made to determine how many unilateral infections developed into bilateral infections. Observations were made and specimens were obtained from cattle in all stages of the disease, from the initial onset to recovery.

The following results were obtained from the examination of the 62 cattle in the Columbus area:

1) Of the 90 infected eyes examined, *H. bovis* was identified by the direct microscopic technique in 66 (73.3%); by the cultural method in 69 (76.6%); and by the combined method in 74 (82.2%).

2) The cultural method and the direct microscopic method were in agreement in 55 instances.

3) Of the 34 apparently normal eyes of the unilaterally infected cattle, 5 were found to contain *H. bovis*.

The majority of colonies cultivated from eyes in the acute ophthalmitis stage of the infection were *H. bovis*. Microörganisms such as staphylococci, streptococci, diphtheroids, and *Pasteurella multacida* as well as *H. bovis* were frequently isolated from chronically infected eyes.

Of the 22 specimens obtained from outside of the state, *H. bovis* was detected by direct microscopic examination in 19. Only twice was it possible to cultivate this organism from the infective material, largely because of the saprophytic contaminants and the time spent in transit.

Morphology and Cultural Characteristics of H. bovis.—The morphology and cultural characteristics of *H. bovis* isolated from infected eyes were studied and found to be the same as described by Little and Jones.[2]

Following are the observations that were made, including some interesting properties not mentioned by Little and Jones[2]:

a) Morphology.—*H. bovis* is a gram-negative short plump rod measuring 0.5 to 1.0 μ in width and 1.5 to 2.0 μ in length. It is regularly found paired end to end. Pleomorphism is exhibited in old cultures. A tendency to bipolar staining is occasionally noted. A well-defined capsule is evident in freshly isolated organisms. It is nonmotile and does not form spores.

b) Cultural Characteristics.—The best growth is obtained on a slightly alkaline

*Specimens from California were received from Dr. Joseph Giambroni, a large animal practitioner at Woodland.

†Specimens from Montana were obtained from several deputy state veterinarians in various parts of the state, through the cooperation of Dr. W. J. Butler, state veterinary surgeon, Helena.

(pH 7.2 to 7.5) solid medium enriched with 5 per cent citrated horse blood. At the end of twenty-four hours incubation at 37 C., the colonies appear greyish-white, regular, convex, and approximately 1 to 2 mm. in diameter. The colonies are firm and tend to break apart when touched with an inoculating needle. Beneath each colony is a depression in the medium due to liquefaction. A clear zone of hemolysis, 1 to 2 mm. in width, occurs around each colony. Colonies older than twenty-four hours become larger and flatter, and have a small central raised area. Growth upon nutrient agar is poor and occurs only when a large inoculum is used.

Liquid mediums, even when enriched with blood serum, do not support a heavy growth. That which is obtained is characterized by the formation of large clumps in the bottom of the tube.

Dextrose, sucrose, lactose, maltose, xylose, mannitol, dulcitol, dextrin, inulin, and salicin are not fermented; nitrates are not reduced to nitrites; indol is not formed; gelatin is slowly liquefied; coagulated rabbit serum is completely lysed in from three to five days; and growth in litmus milk is characterized by an alkaline reaction and the formation of a heavy white casein precipitate in seven to ten days.

Pathogenicity of H. bovis.—(*a*) Mice, guinea pigs, and rabbits were exposed to eye instillations of infective material from cattle, and to large doses of *H. bovis* by various parenteral routes. No ocular reaction was obtained in any of the animals and mice and guinea pigs failed to respond to parenteral injections. Rabbits irregularly succumbed to intravenous inoculations of *H. bovis*. Further investigation will be necessary to interpret these results.

b) Sheep.—The eyes of 2 young sheep were instilled with infective material from cattle and the eyes of 6 were inoculated with a saline suspension of *H. bovis* by injecting as much of the material as possible into the conjunctival sac and scarifying the cornea.

None of the animals reacted to the inoculations and *H. bovis* was not recovered from any of the eyes three days following the injections.

c) Cattle.—Calves of mixed breeds varying from 4 to 8 months of age were carefully selected for the experiments. They were purchased from sources least likely to have been exposed to infectious keratitis.

All animals were kept in a barn throughout the experiments and were observed for a period of six to eight weeks prior to inoculation. Weekly bacteriological exam-

TABLE I—Pathogenicity of Hemophilus Bovis for Cattle During the Summer and Autumn

CALF NUMBER	EYE INOCULATED AND METHOD	CLINICAL REACTION AND INCUBATION PERIOD		DAYS AFTER INOCULATION THAT *H. bovis* WAS CULTIVATED FROM THE EYE	
		RIGHT EYE	LEFT EYE	RIGHT	LEFT
1.	Right; uninjured.	Acute ophthalmitis (mild). Onset in three days.		5	
2.	Right; scarified.	Acute ophthalmitis. Onset in six days.		30	
3.	Both eyes; only the right was scarified.	Chronic ulcerative keratitis. Onset in four days.	Acute ophthalmitis. Onset in six days.	64	51
4.	Both eyes; only the right was scarified.	Acute ophthalmitis. Onset in seven days.	Acute ophthalmitis. Onset in thirteen days.	40	40
5.	Both eyes; only the right was scarified.	Ulcerative keratitis. Onset in three days.	Ulcerative keratitis. Onset in three days.	62	50
6.	Both eyes; only the right was scarified.	None.	None.	0	0

inations were made of the eyes of all cattle, both before and after the inoculation. Throughout the experiments observations were made every two to four days.

Inoculation was done by instilling as much of the bacterial suspension as possible into the conjunctival sac and forcefully holding the eye lids closed for one minute to insure the distribution of the material on all external structures of the eye. Two experiments were conducted:

Experiment 1.—This experiment was made during the summer and the autumn months. Six calves were inoculated as indicated in table I. Scarification was accomplished by traumatizing the cornea and conjunctiva with a hypodermic needle.

Five of the 6 calves inoculated in one or both eyes with *H. bovis* reacted by developing symptoms and lesions indistinguishable from infectious keratitis. The severity of the infection varied from an acute ophthalmitis, lasting only one week and resulting in only superficial ulceration, to a chronic ulcerative keratitis with extensive corneal ulceration and prolapse of Descemet's membrane. The incubation period varied from three to thirteen days. *H. bovis* was found to persist in the eyes of the cattle that showed symptoms for as long as sixty-five days after being inoculated, though the eyes appeared to be completely recovered.

Calf 6 failed to respond to the first and second inoculation with a different strain of *H. bovis*. All bacteriological examinations of the eyes of this animal were negative for *H. bovis*, both before and after being inoculated. Upon investigating the origin of this animal, it was discovered that it had come from a farm on which infectious keratitis was prevalent. It was assumed that a previous infection may have rendered the animal resistant.

Experiment 2.—The experiment was conducted during the winter. Eleven calves were inoculated by the same technique previously described and as indicated in table 2.

Calves 10 and 11 had been previously

TABLE 2—Pathogenicity of Hemophilus Bovis for Cattle During the Winter

CALF NUMBER	EYE INOCULATED AND METHOD	CLINICAL REACTION AND INCUBATION PERIOD		DAYS AFTER INOCULATION THAT *H. bovis* WAS CULTIVATED FROM THE EYE	
		RIGHT EYE	LEFT EYE	RIGHT	LEFT
1.	Right; uninjured.	Conjunctivitis. Onset in five days.		58	
2.	Right; uninjured.	Acute ophthalmitis. Onset in sixteen days.		58	
3.	Right; uninjured.	Acute ophthalmitis. Onset in eight days.	Spontaneous infection. Acute ophthalmitis. Onset sixteen days after the right eye was inoculated.	147	
4.	Both; uninjured.	Acute ophthalmitis. Onset in seven days.	None.	27	27
5.	Both; uninjured.	Acute ophthalmitis. Onset in three days.	Acute ophthalmitis; onset in nine days.	78	78
6.	Both; uninjured.	None.	None.	58	41
7.	Right; scarified.	None.		41	
8.	Right; scarified.	Acute ophthalmitis. Onset in fifteen days.	Spontaneous infection. Acute ophthalmitis. Onset in thirty-five days after right eye was inoculated.	78	
9.	Right; scarified.	Acute ophthalmitis. Onset in thirteen days.		78	
10.	Both; scarified.	None.	None.	0	0
11.	Both; scarified.	None.	None.	0	0

inoculated with *H. bovis* in experiment 1 and were numbers 4 and 5, respectively, of that experiment. They had completely recovered, other than from corneal scars. For a period of ninety-five days prior to the second inoculation no *H. bovis* was found. The object of the second inoculation of these 2 animals was to determine if one infection rendered the animals immune. The other 9 animals were inoculated for the first time. The results are given in table 2.

Symptoms were observed in one or both eyes of 7 of the 9 animals inoculated for the first time. They appeared in from five to sixteen days after the inoculation. The majority of the calves that reacted to the inoculation showed symptoms typical of the sudden onset of infectious keratitis, but failed in all instances to develop into the chronic ulcerative type produced in some of the animals inoculated during the summer.

Infection was not observed in calf 6, but a minute corneal scar developed in one eye. Since daily observations were not made, a mild infection could have gone unobserved. Neither calf 10 nor calf 11 was observed to develop an infection and in no instance was *H. bovis* recovered from their eyes.

Bacteriological examinations of the eye secretions of the animals inoculated for the first time revealed *H. bovis* in all the eyes inoculated and in the eyes that became spontaneously infected. Even though no evidence of infection other than corneal scars existed forty days after inoculation, the inoculated eyes and spontaneously infected eyes of the calves inoculated for the first time contained *H. bovis*.

SEROLOGICAL STUDIES

Blood samples were drawn from the 11 calves used in experiment 2, ten days before and sixty days after inoculation. The blood serum was tested for the presence of bacteriolytic and precipitin antibodies. No antibodies were detected.

Agglutination tests were not conducted because no method was found whereby the spontaneous agglutination of *H. bovis* could be inhibited.

DISCUSSION

The terms "infectious keratitis" and "pinkeye" are used almost exclusively by veterinarians when referring to the disease described. The clinical and bacteriological picture resembles so closely that of infectious ophthalmia, investigated by Little and Jones[2,3] that it is probably the same disease.

The similarity of the bacteriological findings in native cattle, cattle shipped into the Ohio area from the various western states, and in specimens submitted from infected cattle in California and Montana indicates that infectious keratitis is a widespread specific disease probably caused by *H. bovis*. Further proof that this organism is the cause is its ability to induce an identical infection in susceptible cattle experimentally.

Farley[5] was unable to reinfect animals that had recovered from acute or chronic infections with virulent eye secretions. Similarly, 2 calves that had recovered from chronic infections induced by *H. bovis* were not susceptible when exposed the second time to that organism. Further work upon the specificity and duration of immunity is certainly indicated. Since systemic antibodies were not demonstrated in the 11 calves tested, the resistance to infection is probably due to a local tissue immunity.

Three calves (calf 6 of experiment 1, and calves 6 to 7 of experiment 2) resisted infection upon their first exposure to *H. bovis*. A similar proportion of cattle used by Farley[5] resisted infection when inoculated with virulent eye secretions.

The persistence of *H. bovis* in the eyes after recovery, as shown in experimentally infected cattle, is possibly the means by which infectious keratitis is carried over from one season to the next.

Sheep are susceptible to an eye disease which is clinically similar to bovine infectious keratitis. The failure of sheep to react to inoculations of either virulent eye secretions of cattle or to *H. bovis* indicates definitely that the eye disease in sheep is caused by some other agent.

SUMMARY

The investigational findings indicate that *Hemophilus bovis* is the probable cause of infectious keratitis.

Specimens for bacteriological study were obtained from native cattle, feeder cattle, and cattle in California and Montana. *H. bovis* was found in 93 (83%) of 112 infected eyes examined. It was not found in either eye of 20 normal cattle. Cultures of *H. bovis* when inoculated into the conjunctival sac, induced an infection indistinguishable from infectious keratitis in one or both eyes of 12 of 15 animals. Injuring the cornea and conjunctiva did not seem to be necessary to induce an infection. *H. bovis* persisted in the eyes of the majority of the experimentally infected cattle for two or more months following inoculation, even though the cattle had apparently completely recovered from the infection.

Systemic antibodies were not detected, either before or after inoculation, in the serums of 11 experimental calves by precipitin or complement fixation tests.

Sheep were not found to be susceptible to inoculations of either virulent eye secretions of infected cattle or cultures of *H. bovis*. This fact strongly suggests that the disease of sheep is caused by a different agent.

Mice, guinea pigs, and rabbits were not susceptible to *H. bovis*.

ACKNOWLEDGMENT

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Bloat in Cows on Alfalfa Pasture

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PREVIOUS STUDIES have indicated that bloat can be produced easily by pasturing, at appropriate stages of development, pure stands of alfalfa supplied with optimal amounts of water.[2, 6, 7] These same studies have given information on the effect of supplemental feeding on the incidence of bloat on alfalfa pasture. Although the feeding of grain just before pasturing shortened the time interval between the beginning of pasturing and the onset of bloat, it did not appreciably affect the incidence or severity of bloat. The overnight feeding of Sudan hay completely prevented bloat on alfalfa pasture the following day, but alfalfa hay was less effective. Barley straw was ineffective for lactating dairy cows, probably because of the small amounts consumed. This assumption is substantiated by quantitative studies on Sudan hay, reported herein.

In this paper, data are submitted on: rumen pressure at various stages of bloat, the amount of Sudan hay necessary to prevent bloat, the relation of supplemental hay feeding to the amount of pasture alfalfa consumed, and the insensible losses of body weight * during the pasturing period.

EXPERIMENTAL

Lactating Holstein-Friesians and Jerseys were used. All are indicated by herd numbers through which the breed can be identified: those in the 1,000 series are Holstein-Friesians; 700 to 800 series, Jerseys. For the most part the only animals used were known from previous studies to be susceptible to bloat. None of the animals showed a tendency to bloat on ordinary hay and grain rations, but one animal (759) did bloat once when forced on a high grain-low hay ration (see table 1, Mead, Cole and Regan[7]).

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*The "insensible loss," also called "insensible perspiration," is the total loss of body weight in a given period minus the "sensible loss," namely, the weight of feces and urine excreted.

Three three-acre plots, of alfalfa were used. With this size plot we have found that 8 to 10 cows can be pastured satisfactorily for seven to ten days. If more animals are used the pasture may become too stemmy for such an interval. It is quite important that all parts of the field be fairly uniform in growth. When parts of the field are drier than others, cattle have a tendency to crop the drier parts closely before eating the more tender plants in the wetter portions of the field. The length of time that a field can be used depends also upon the temperature. When the weather is very hot the plants rapidly become wiry, and unsuitable.

As in previous trials, the animals were under constant observation while on pasture. In the feed consumption studies, the animals were weighed before and after pasturing. In the interim, all excreta were collected and weighed. In determining insensible losses, the above data were obtained and the animals were muzzled while in the field to prevent eating.

Rumen Pressure at Various Stages of Bloat.—It is desirable to express the degree of tympany in absolute rather than empirical terms. One means, at our disposal, is to measure rumen pressure. In a few instances[7] the pressure was determined directly by tapping the rumen with a trocar canula and connecting the canula to a mercury manometer. Since this is not a desirable practice as a routine procedure, Kleiber has devised a tympanometer for determining the pressure without tapping the rumen. This instrument measures the thrust necessary to force a stiff plate into the plane determined by a ring pressed to the bulging left flank of the bloated cow. This force is the product of the plate area and the intrarumen pressure and is theoretically independent of thickness and elasticity of the skin. The test of this theory, the construction of the tympanometer and its calibration have been described[4].

Table 1 shows the relationship between the empirical terms we have used to designate different stages of bloat, determined largely by palpation and pressure, and pressures as measured with the tympanometer. The mean pressure is the average of 10 readings taken within a minute. In most instances we actually took many more readings, but there seemed to be no advantage in including more than 10 for the averages.

Severe bloat, as we have defined it, is always accompanied by evident distress: labored

TABLE 1—Rumen Pressure (mm.Hg.) of Several Cows in Various Stages of Bloat as Determined with the Tympanometer

Stage of Bloat	Cow	Range in Pressure	Mean Pressure	Stage of Bloat	Cow	Range in Pressure	Mean Pressure
Gassy	759	6.5 to 15.5	10.0	Marked	792	28.5 to 36.5	32.0
	759	9.5 to 15.5	13.5		1046	38.5 to 46.5	42.0
	1038	13.5 to 29.5	18.5		759	35.0 to 40.0	37.0
	792	14.5 to 25.0	17.5		759	46.5 to 57.0	50.5
Slight	759	16.5 to 20.5	19.0		792	36.0 to 44.0	39.0
	792	23.0 to 30.5	27.0		1046	31.5 to 39.0	35.0
	722	28.5 to 36.5	31.5	Severe	1046	54.0 to 63.5	59.0
	722	29.5 to 36.0	32.5		1046	54.0 to 60.0	56.5
	722	28.5 to 37.5	33.0		1046	55.0 to 61.0	57.5
	1046	36.0 to 44.0	38.5		757	40.0 to 51.0	45.5
	1046	27.0 to 34.0	29.5		757	46.5 to 59.5	51.5
	792	16.5 to 27.5	19.5		759	57.5 to 65.5	62.0
	1038	29.5 to 42.0	33.5		759	58.5 to 69.0	63.5
	1038	29.5 to 44.0	34.0		1046	44.0 to 56.0	48.5
	1003	25.0 to 36.0	29.5		759	57.5 to 61.0	59.5
	792	25.0 to 37.5	31.5				

breathing, frequent urination and defecation, restlessness, and sometimes frothing at the mouth. As seen in the table, there is considerable variation in pressure at which these distressing symptoms occur in different individuals. Cows, almost invariably, stop eating when markedly bloated or at the "gassy" stage. The animals which continue to eat until markedly bloated are more likely to bloat seriously than those which stop in the initial stages.

The pressure of 59.5 mm. Hg. recorded for cow 759 was obtained sometime after she began staggering and just before she went down. In fact, she had gone down once but regained her feet with assistance. This pressure probably represents closely the rumen pressure at the time of death, since the pressure had remained fairly constant for forty minutes prior to this time. Olson[4] reports pressures of 40 to 125 mm. Hg. in 15 animals dying from bloat with an average of 66 mm. Hg. In 8 living bloated animals the pressure was 50 to 70, with an average of 58 mm. Hg. There are, undoubtedly, considerable variations in the pressure which different individuals can withstand. Also, the length of time an animal is forced to withstand a given pressure is probably an important factor in determining whether the pressure is fatal. We have seen animals become progressively more distressed even though the pressure remained constant.

Whether the pressure ever reaches the higher figure of Olson's range, 125 mm. Hg., in *living* animals is speculative. It is true that the pressure will increase from standing to lying but how marked an effect position exerts has not been determined.

Table 1 shows a range in pressure of approximately 10 mm. Hg. when a series of pressure readings are made in rapid succession. This range is due mainly to rumen motility.

Amount of Sudan Hay Necessary to Prevent Bloat.—Two different fields were used in these studies: field 2 from August 30 to September 5, and field 4 from September 19 to September 27. On September 1, 2, 3, 22, and 23, hay was fed at 5 a. m. and the remainder weighed back at 7 a. m. On other

TABLE 2—Quantitative Data on the Prevention of Bloat with Sudan Hay. Eight Cows Were Used Throughout

Date (1944)	Average Pounds of Sudan Hay Consumed	Number of Cows Bloating	Designation of Cows Bloating and Severity of Bloat
Aug. 30	None	4	757-d, 759-m, 792-m, 1003-d.
Aug. 31	None	5	757-d, 759-d, 792-d, 1003-d, 1038-d.
Sept. 1	4.4	2	759-d, 792-d.
Sept. 2	5.4	4	757-m, 759-d, 1003-d, 1038-m.
Sept. 3	7.7	2	757-d, 759-d.
Sept. 4	None	5	722-d, 757-d, 759-d, 792-m, 1003-d.
Sept. 5	None	5	722-m, 757-d, 759-d, 792-d, 1046-d.
Sept. 19	None	1	759-s.
Sept. 20	None	3	792-d, 1038-d, 1046-s.
Sept. 21	None	5	759-d, 792-m, 1003-d, 1038-m, 1046-d.
Sept. 22	10.6	2	759-d, 1046-d.
Sept. 23	9.3	2	757-s, 759-d.
Sept. 24	14.2	1	722-d.
Sept. 25	None	5	722-d, 759-s, 792-m, 1003-d, 1030-d.
Sept. 26	17.7	None	
Sept. 27	None	8	722-d, 757-d, 759-d, 792-d, 1003-d, 1030-d, 1038-d, 1046-d.

d = definitely or slightly bloated; m = markedly bloated; s = severely bloated.

dates of hay feeding, the cows had access to limited amounts of hay overnight.

Not until 17.7 lb. of Sudan hay had been consumed the night preceding pasturing, was bloat completely prevented (table 2). On September 24, when 14.2 lb. of Sudan was fed per cow, no cows bloated during the pasturing period, but 1 cow (722) bloated slightly following the feeding of grain after removal from the field. Under our conditions, cows would not eat sufficient hay during a two-hour period just before pasturing to prevent bloat. Only by giving access to all the hay they would consume overnight was bloat completely prevented. It is true that a rather poor quality of hay was used; possibly cows might be fully protected if they were given access to a better hay for two hours.

Earlier as well as recent studies indicate that alfalfa hay is not satisfactory in preventing bloat. In one trial run this past summer (October '44), an average of 11.2 lb. of alfalfa hay per cow was given to 18 cows overnight. Nine of the 18 cows bloated the following day on alfalfa pasture as compared to an average of 8 cows bloating on the same pasture during three preceding days when no hay was fed. Neither was there an appreciable reduction in the severity of bloat when alfalfa hay was fed. This was an exceptionally fine quality of hay and better prevention previously has been obtained with coarser hay[2].

The relatively large amounts of Sudan hay necessary to prevent bloat, and the failure of alfalfa hay in this respect, are distinct limitations in the value of hay feeding in bloat prevention. In spite of these limitations, however, the feeding of Sudan hay is still one of the best known preventive procedures. Mixing of grasses with legumes is perhaps the most satisfactory procedure under most conditions but this method also has its limitations. In certain seasons it may be difficult to maintain sufficient grasses in the mixture to prevent bloat while, on the other hand, grasses may predominate at other times and lower the yield of the pasture. Pasturing on Sudan grass overnight with pasturing on alfalfa the following day[2] would appear to be a practical procedure under many conditions and further attention should be given this method. Also the use of other roughages in preventing bloat needs investigation. Jacobsen, Espe and Cannon[3] reported, for example, that silage inhibited fermentation of rumen ingesta *in vitro;* studies with living animals should also be made.

TABLE 3—Insensible Losses While on Alfalfa Pasture (Cow 757)

DATE (1944)	TIME BETWEEN INITIAL AND FINAL WEIGHT MINUTES	INITIAL WEIGHT (LB.)	FINAL WEIGHT (LB.)	LOSS IN WEIGHT (LB.)	EXCRETA (LB.)	INSENSIBLE LOSS (LB.)	INSENSIBLE LOSS PER HOUR (LB.)
Sept. 22	215	824	807	17	9	8	2.24
Sept. 23	157	823	806	17	8.1	8.9	3.4
Sept. 24	163	825	809	16	7.4	8.6	3.3
Sept. 25	168	795.5	774.5	21	13.7	7.3	2.6
Sept. 27	148	761	748.5	12.5	8.3	4.2	1.7

Feed Consumption Studies.—The amount of rumen gas formed is directly related to the amount of feed consumed[1]. A knowledge of feed consumption is therefore advantageous in bloat studies. In fact, it would be desirable to have a knowledge of the feed consumption of each animal on test. Under present conditions, such an ambitious program is not feasible, but limited information on food intake has been obtained.

In our previous studies[7] on consumption of pasture alfalfa, only the sensible losses in body weight were taken into account. As our present studies were conducted during hot weather, it seemed desirable to determine the magnitude of insensible losses in body weight. These losses were determined for cow 757 on five days (table 3). She was muzzled and turned into the field for a two-hour period (September 22, the period was longer) and handled in the same manner as when feed consumption data were being collected. The initial body weight was determined; from this moment until the final weight was obtained, all excreta were collected and weighed. The animal was allowed to roam over the alfalfa pasture with seven other cows on a bloat experiment; a halter with a long lead rope restrained her when excreta were being weighed. The cow was very tractable and displayed no nervousness because of being muzzled. Thus, the losses shown by this animal should be fairly characteristic of normal grazing animals. The time between initial and final weights was greater than two hours because of the time taken to go to and from the field to the scales.

On September 22 to 25, inclusive, the experimental period in the field was from 10 a. m. to 12 noon. During this period, the insensible losses ranged from 2.2 to 3.4 lb. per hour. On September 27, the experimental

period was from 8 to 10 a. m. During this cooler part of the day, the insensible loss was 1.7 lb. per hour. On this date, which was fairly characteristic for the entire experimental period, the temperature in the alfalfa field in the sun one meter above the ground was 16 C. at 8 a. m., 24.7 C. at 10 a. m., and 34 C. at 12:30 p. m. The corresponding figures for the relative humidity were 97, 77, and 53 per cent, respectively. The greater insensible loss in the warmer part of the day is due, in part, to increased respiratory rate with consequent increased vaporization.

the first 2.7 hours and 2.9 lb. per hour for the remaining four hours.

Cow 757 ate an average of 18.5 lb. when she received no hay as compared to 22.1 lb. when hay was fed the night before pasturing; ignoring insensible loss, the average figures are 13.2 and 16.1 lb. respectively. In similar studies made a year ago[7] in which insensible loss was ignored, the same cow, while dry and receiving 6 lb. of concentrates but no hay, ate an average of 10.8 lb. of green alfalfa in a two-hour period. Last year's studies, in which feed consumption was determined simultane-

TABLE 4—Amount of Alfalfa Consumed by Lactating Cows on Pasture for Two or Six Hours with and Without the Supplemental Feeding of Sudan Hay

		Cow 757		Cow 759 or 722§		Cow 722
Date (1944)	Average* Sudan Hay Consumed (Lb.)	Amount Consumed† in Two Hr. (Lb.)	Bloat During Test‡	Amount Consumed in Two Hr. (Lb.)	Bloat During Test	Amount Consumed in Six Hr. (Lb.)
Aug. 31	None	12.3 (16.9)	None	14.7 (19.3)	d	.....
Sept. 1	4.1	12.8 (17.4)	None	10.7 (15.3)	d	.....
Sept. 2	5.5	16.1 (20.7)	d	19.5 (24.1)	d	.....
Sept. 3	7.2	13.9 (18.5)	None	.....	.....	.....
Sept. 4	None	14.6 (19.2)	d	18.0 (22.6)	d	.....
Sept. 5	None	12.7 (17.3)	g	12.8 (17.4)	d	.....
Sept. 19	None	.....	.....	17.2 (21.8)	None	33.1 (49.3)
Sept. 20	None	6.7 (11.3)	None	20.9 (25.5)	None	31.2 (47.4)
Sept. 21	None	15.3 (19.9)	None	7.7 (12.3)	None	22.7 (38.9)
Sept. 22	10.0	14.5 (22.3)	None	8.1 (12.7)	None	28.3 (44.5)
Sept. 23	9.7	20.2 (28.0)	None	5.1 (9.7)	None	18.7 (34.9)
Sept. 24	14.2	15.3 (23.1)	None	8.3 (12.9)	None	22.0 (38.2)
Sept. 25	None	17.7 (26.1)	g	26.7 (31.3)	d**	15.4 (31.6)
Sept. 26	17.7	20.1 (24.7)	None	7.5 (12.1)	None	16.4 (32.6)
Sept. 27	None	.....	.....	19.1 (23.7)	g	31.0 (47.2)

*Individual hay consumption was determined on Sept, 1, 2, 3, 22, and 23. In no instance did the consumption by the 2 cows vary more than 0.6 lb.

†The figures outside parenthesis refer to the consumption of green alfalfa determined without taking insensible loss of body weight into account; those inside parenthesis were calculated on the basis of both sensible and insensible losses. *See* text for details of computation.

‡Symbols for bloat: g = gassy, d = definite or slight bloat.

§Cow 759 on test Aug. 31 to Sept. 5; cow 722 from Sept. 19 to 27.

**Bloat occurred after the first two-hour test, thus explaining why she ate practically nothing during the remainder of the day.

The data on feed consumption are shown in table 4. All cows on test received 6 to 8 lb. of a concentrate mix daily. The manner of handling the animals was the same as that for the determination of insensible loss described above except that the animals were not muzzled. Figures calculated both with and without considering insensible loss are given. Feed consumption and insensible loss obviously cannot be determined concurrently. The data on insensible loss in table 3 were used, therefore, for calculating feed consumption. For two-hour trials (2.7 hours between weighings) conducted between 8 and 10 a. m., the insensible loss figure obtained on September 27 (table 3) was used. In other words, 4.6 lb. (1.7 lb. per hour for 2.7 hours) was assumed to be the insensible loss. For two-hour trials conducted between 10 and 12 a. m. (a hot part of the day) the average figure for September 22 to 25, inclusive (table 3) was used (2.9 lb. for 2.7 hours). For six-hour trials (6.7 hours between weighings) the calculation of insensible loss was on the basis of 1.7 lb. per hour for

ously for a dry and a lactating cow on the same field, indicated that lactation had a more pronounced effect on feed consumption than is indicated by comparing the results on the same cow for two different years. It is probable that the alfalfa in this year's studies was younger and consequently less palatable than that used last year.

Cow 759, on test August 31 to September 5, consumed 19.8 lb. of green alfalfa without hay in a two-hour period as compared to 19.7 lb. when receiving 4.1 to 7.2 lb. of Sudan hay the night prior to pasturing.

Cow 722, on test September 19 to 27, consumed an average of 22.9 lb. without hay and 11.8 lb. when receiving 9.7 to 17.7 lb. of hay.

All 3 animals consumed, on the average, 20.3 lb. without supplemental feeding of hay, and 18.6 lb. when hay was fed. Thus, the results indicate that the total consumption of feed is greater for cows on alfalfa pasture supplemented with hay than for cows on alfalfa pasture without hay. In certain animals, the total intake is augmented not only by the

hay consumed but also by increased consumption of green alfalfa.

The question arises as to how the consumption of alfalfa during the first two hours on pasture compares with that for a six-hour period (approximately the length of the daily pasturing period for our dairy herd). This comparison is shown for cow 722 in table 4, an average of 18.0 lb. for the two-hour period as against 40.5 for six hours.

In computing insensible losses for cows 759 and 722, we have not taken into account the fact that these cows were approximately 200 lb. heavier than cow 757 on which insensible losses were determined. Assuming that the insensible loss increases according to increases in metabolic body size,[5] the values on feed consumption for the six-hour period given for cow 722 in table 4 should be increased by approximately 3 lb.

DISCUSSION

Our studies on the bloat problem on alfalfa pasture have extended over a three-year period. Some of the same animals have been used throughout. Bloat has been easily produced each season. It is difficult to say how many of the bloat cases would have been fatal without treatment because in most instances the animals used were too valuable to postpone treating until they had become seriously bloated. Some less valuable animals have been allowed to develop more serious bloat. One animal (1046, table 1) became severely bloated and developed a mean rumen pressure ranging from 57 to 59 mm. Hg. At this pressure, she was in distress as evidenced by labored breathing, frequent urination and defecation, and frequent attempts to lie down, which uneasiness averted. Just an hour elapsed from the time she was definitely slightly bloated until the bloat was severe. In another fifteen minutes, however, she began belching and was soon improved without treatment. Other animals have shown marked distress at pressure as low as 27 mm. Hg. One cow (759) did reach the stage of prostration. She had fallen down once but with our assistance regained her feet, and a stomach tube was inserted. A review of events up to and at the time of bloating follows:

Regular grain allowance night and morning.
No hay fed.

7:52 a. m.—Turned on alfalfa pasture. Eating.
8:27 a. m.—Drinking.
8:28 a. m.—Eating again.
8:49 a. m.—Stopped eating.
9:02 a. m.—Eating.
9:34 a. m.—Stopped eating. Gassy.
9:47 a. m.—Slightly bloated.
10:23 a. m.—Markedly bloated. Rumen pressure, 37 mm. Hg.
10:57 a. m.—Almost severely bloated. Rumen pressure, 51 mm. Hg.
11:04 a. m.—Severely bloated. Rumen pressure, 55 mm. Hg. Frequent urination and defecation. Gave 2 oz. turpentine.
11:13 a. m.—No improvement. Rumen pressure, 58.5 mm. Hg.
11:17 a. m.—No improvement. Rumen pressure, 56.5 mm. Hg. Gave 3 oz. turpentine.
11:30 a. m.—No improvement. Gave 2 oz. turpentine.
11:37 a. m.—Rumen pressure, 59.5 mm. Hg. Lay down but got up immediately.
11:47 a. m.—Makes movement to lie down but does not. Rumen pressure, 59.5 mm. Hg. Staggers.
11:47 a. m.—Falls down. Cannot drive her up.
11:50 a. m.—Finally forced her to stand up. Inserted stomach tube.

Gas pocket was immediately tapped. At 12 noon she appeared entirely normal.
She was kept on pasture until 2:50 p. m., but did no further eating.

This animal, which almost certainly would not have recovered without treatment, had approximately the same rumen pressure as the cow, previously cited, which recovered spontaneously. This is the first animal which failed to respond to turpentine, but treatment was withheld for over an hour after she was definitely bloated.

Although we can produce bloat easily and have had many seriously bloated animals, it must be admitted that we have not been able to seriously bloat, regularly, a majority of animals on a given day. To produce serious bloat it is essential that the animals consume a large amount of alfalfa. A study of factors affecting the consumption of pasture alfalfa is, therefore, indicated. Although we do not have sufficient data to prove the point, the impression we gained is that alfalfa in the younger stages was relatively unpalatable. There can be no doubt that alfalfa grown under wet condi-

tions is relatively unpalatable for we have repeatedly seen animals cropping off the alfalfa on the drier parts of the field first. Studies on the palatability of alfalfa grown under different conditions are indicated as well as further studies on the effect of supplemental feeding upon the consumption of alfalfa pasture.

SUMMARY AND CONCLUSIONS

1) By means of a special instrument called a tympanometer, rumen pressure at various stages of bloat was determined without tapping the rumen. Animals with a rumen pressure of 60 mm. Hg. are in a serious condition.

2) Four to 7 lb. of Sudan hay fed two hours preceding pasturing were ineffective in preventing bloat. Bloat was only prevented when animals had access to all the Sudan hay they would consume overnight prior to pasturing on alfalfa. Seventeen lb. of Sudan hay completely prevented bloat.

3) There were individual differences in the influence of supplemental feeding of Sudan hay upon the amount of alfalfa pasture consumed; in some instances hay feeding increased consumption, in others, depressed it. Considering the results for the past two years it appears that the supplemental feeding of Sudan hay increases consumption of green alfalfa if no concentrates are fed. When both concentrates and Sudan hay are fed, most cows eat less alfalfa than if only concentrates are given.

4) Insensible losses during hot weather are appreciable—amounting to about 3 lb. per hour in an 800-lb. cow.

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Nitrite Poisoning from Ingestion of Plants High in Nitrate

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POISONING from ingestion of plants high in nitrate content has been reported, most cases being confined to cattle. It is of interest to note that in most cases where careful records are reported, the poisoning was associated with a recent rain, snowfall, or dampness of the stack. In some cases, the hay had been fed for varying periods before the dampness occurred, or was fed to experimental animals a week or more after losses occurred. In either case, the hay failed to cause poisoning.[1]

Symptoms of nitrate poisoning in cattle and horses have been produced by feeding potassium nitrate in large doses.[2] Guberlet[3] has reported nitrate poisoning in chickens, caused by ingestion of that salt.

Nitrate as such is relatively nontoxic, but is capable of reduction to nitrite. Nitrite is readily absorbed from the digestive tract, combines with hemoglobin to form methemoglobin, and produces symptoms similar to cyanide poisoning.

During the second week of November, 1944, turkeys from 4 widely separated flocks in Cache and Bear River Valleys, Utah, were brought into the veterinary laboratory of the State Agricultural College for diagnosis. Two flocks had been grazing on oat stubble fields, 1 on a wheat stubble field, and 1 on an uncultivated field. All four fields contained pigweed, stunted grasses, thistles, various other weeds, and some sprouting grain under the stubble or around feeders. Three flocks were being watered on irrigation ditches and 1 with well water in troughs. All had dry mash before them at all times. The flock history and symptomatology were similar in all cases. The flocks had been doing well, and were apparently free of disease except for some sinusitis. There had been no rain for approximately two months previous to the day before trouble appeared in these flocks. A heavy rainfall ended the drought and the day following the rain, feed consumption of the birds fell off to 10 per cent of what it had been. The birds showed a diarrhea, the feces being almost water clear, with bright green plugs and flakes. All birds in all flocks showed some degree of cyanosis, varying from deep purple to slight changes from normal, and all exhibited varying degrees of depression. Mortality ranged from 0 to 12 per cent. Birds which survived forty-eight hours showed gradual improvement. Anorexia, cyanosis, and diarrhea disappeared from the flocks in from eight to ten days. Weight loss in all flocks was extremely serious because of the prolonged symptoms.

Because flocks 60 miles apart were affected simultaneously, and all birds of each flock were affected the same day, poisoning was suspected. Nutrient, agar-slant cultures from the liver, spleen, and heart blood of typically affected birds in each flock were negative. Gross pathological changes were not constant. From none to one or more of the following lesions were seen: dehydration, mucoid enteritis, hyperemia of intestinal mucosa, petechiae in the epicardium, dark blood, and pinpoint degenerated areas on the liver surface.

Whole blood, blood serum, and crop and gizzard contents of birds from each flock were tested for cyanide by the picrate paper method and results were negative. All gizzards were filled with bright green material, a mixture of weeds, grasses, and some mash. Samples of each type of plant found on two of the four fields were gathered five days after symptoms were first noted, and were tested for cyanide with negative results.

Because of negative cyanide tests, the same plants were then tested for the pres-

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From the Agricultural Experiment Station, Utah State Agricultural College, Logan.

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ence of nitrite by the sulfonilic acid-alphanapthylamine qualitative test. All specimens were negative for nitrite. No nitrate determinations were made. Feces and blood of 4 typically affected birds were tested for nitrite. Positive tests were obtained from 3 of the birds from both feces and blood, and traces of nitrite were detected in the fourth bird. Feces and blood of normal healthy birds raised in houses and running on wire floors were negative for nitrite.

To observe the effects of nitrate on a turkey, a healthy 10-lb. bird was given 50 gr. of potassium nitrate and observed. A watery diarrhea was evident in about six hours after dosing. No cyanosis was observed and the appetite remained good. Three days later the bird was apparently normal. It was then given an oral dose of 3 gr. of sodium nitrite in 5 cc. of distilled water. A slight cyanosis was evident in one hour and other symptoms typical of birds in affected flocks appeared. Blood serum and feces were tested the day following nitrite administration and both were positive to nitrite tests. A second dose of 4 gr. of sodium nitrite in water was given after the tests were made, and the bird died in two hours.

A diagnosis of nitrite poisoning was made on the basis of finding nitrite in the blood and feces of affected birds, negative results of nitrite tests in normal birds, absence of evidence of bacterial disease or cyanide, and reproduction of the syndrome by administering nitrite salt to a healthy bird.

If nitrite poisoning in the turkey flocks was caused by ingestion of large amounts of plant material high in nitrate, with the subsequent reduction of nitrate to nitrite in the digestive tract, why did the poisonings occur in several flocks simultaneously and not until after a rainstorm which ended a long drought period? Flock owners stated that the birds, as far as they had observed, had not changed feeding habits during the rain but had been eating plants and weeds on ditchbanks, and scratching in the dry stubble for seeds and sprouts, previous to the poisoning. At least two explanations of the relation between recent moistening of the plants and toxicity are possible. The first is that when dry and dusty the sprouts and leaves were unattractive to the birds and were eaten only in small quantities, furnishing subclinical doses of nitrate to each bird. A rain washed the dust from the plants and made them more attractive so that greater amounts of nitrate-rich plants were eaten. Bradley, Eppson, and Beath[2] fed sublethal doses of potassium nitrate to cattle for periods ranging from one week to two months, and failed to cause either chronic or acute poisoning. Subsequent dosage with lethal amounts of KNO_3 produced acute poisoning. If this explanation is correct, why have there been cases where cattle have been eating from an oat-hay stack with no ill effects and then become poisoned from eating the same hay after a rainfall, the hay being nontoxic to experimental cattle a week or more later? Total amounts of hay consumed would be approximately equal before, during, and after a rain or snow.

The second possible explanation of toxicity of plants rich in nitrate might afford a clue as to why poisoning occurs following dampening of such plants. That explanation, briefly, is this: Dry plants, abnormally high in nitrates and containing no nitrites may contain a reducing substance which reduces nitrates to nitrites and then to ammonia, the reduction taking place when the reducing substance is activated by water, air, and the proper pH. The reduction would go to completion in a period of two or three days, leaving the plants with less nitrate, little or no nitrite, and no ammonia. During a limited period following a rain or snow on dormant or stunted nitrate-rich grass or hay, nitrites might be present in amounts large enough to cause acute poisoning. There is evidence to be found in botanical and agronomical research work that such a reduction occurs microchemically in normal plants, and might occur to a greater degree under abnormal field conditions.

J. K. Wilson[4] points out that oats grown in a greenhouse may contain 14.4 per cent

nitrate (dry weight) calculated as KNO_3, and that weeds grown in open fields contain from traces to 8.5 per cent nitrate. Olsen and Whitehead[5] point out that a toxic lot of pigweed contained 6.01 per cent nitrate and 20 samples of oat-hay ranged from 0.41 per cent to 5.99 per cent dry weight of nitrate, averaging 3.15 per cent when grown in "fairly dry sections." Oat, barley, and wheat hays and pigweed have all been incriminated in nitrate poisoning.[6] Nitrate levels high enough to be toxic probably represent abnormal physiological states of these plants, but such states do occur in nature. Miller,[7] in his textbook on plant physiology, gives an extensive bibliography and a summary of the evidence of the presence in plants of a reducing substance which reduces nitrates to nitrites and then to ammonia in the normal synthesis of plant proteins.

Eckerson[8] has shown, by drying and powdering tomato plants, that the reducing substance is stable, withstands drying, and is reactivated when put back into solution. The substance will reduce plant nitrates, or nitrate salts put in a solution with it. Eckerson conducted an experiment on tomato plants which demonstrated that, under certain conditions, plants may contain appreciable amounts of nitrites, while normally they contain only traces or no nitrite at any given time. In her experiments, plants were grown under nitrate-free conditions and tested for nitrate, nitrite, and ammonia. All tests were negative. The plants were then fed calcium nitrate via soil. Within twenty-four hours nitrates were present in all parts of the plants. The tops of a few plants gave a slight reaction for nitrite and there was no reaction for ammonia. Within thirty-six hours, all plants had considerable nitrite in various parts of the plants, associated with traces of ammonia. Within forty-eight hours, there was slightly less nitrite but more ammonia. Within three to five days, there was very much less nitrite. After the first five to ten days there was little or no detectable nitrite. In test tubes or beakers, the plant-reducing substance required free access to air in order to bring about reduction of nitrates. The optimum pH for the reduction is slightly alkaline, pH 7.6. Acid prevents the reaction.

A field plant or hay stack with an abnormally high nitrate content and in a dormant state might be compared with Eckerson's plants twenty-four hours after the addition of nitrate to the soil. Rain or melting snow might activate the reduction of nitrate to nitrite, bringing about a period of twelve to twenty-four hours in which the plant or stack would have a relatively high nitrite content, which would render the material highly toxic. To test this theory, the following experiments were conducted:

EXPERIMENT 1

1) *Problem:* to determine whether or not dry, nontoxic, nitrite-free oat hay, after being subjected to moisture, may contain nitrite.

Method: A sample of all parts of oat hay which was being fed to cattle and horses with no ill effects was obtained from a barn loft, cut into 1/8 inch pieces, and placed in test tubes in 1-Gm. lots. Sufficient distilled water was then added to the hay to cover it. In some series of tests, the hay was left under water, and in others the hay was scraped up onto the sides of the tubes so it would remain moist but exposed to air. Three 1-Gm. lots were tested quantitatively for nitrate calculated as sodium nitrate.*

Samples were then tested for nitrite at two-hour intervals. Controls of distilled water were run with each series of samples. Duplicate tests on finely ground samples of the same hay were run. Duplicate tests were run on turkey mash containing 20.0 per cent alfalfa meal. *Results:* The oat hay used in the experiment contained 0.53 per cent nitrate, calculated as sodium nitrate. Nitrite tests in all controls were negative. At fourteen hours, traces of nitrite were detected in samples exposed to air. Samples under water were negative.

The amount of nitrite present increased to a peak in from eighteen to twenty-two hours. Tests were then strongly positive in samples exposed to air, faintly positive in those under water. At sixty hours, the turkey mash samples were negative; oat hay was still positive; but the nitrite was less concentrated than at twenty-two hours.

In the tests conducted with the finely ground hay, the time factor for nitrite production was the same as in samples with larger pieces of hay, but a greater amount of nitrite was produced.

*Nitrate tests conducted by James Thorne, U. S. Soil Conservation Service, Logan.

Four 1-Gm. samples of ground oat hay were tested quantitatively for nitrite, after twenty hours, by comparing with standards of known strength. All assayed 0.22 per cent $NaNO_2$.

Conclusions: Oat hay, when moistened with distilled water, and in the presence of air, will convert its nitrate to nitrite. The maximum amount of nitrite is present eighteen to twenty-two hours after the hay is moistened. Slightly less than 1/2 the nitrate present in the dry hay was converted to nitrite and present as such at twenty hours.

EXPERIMENT 2

2) *Problem:* To determine whether or not dry oat hay containing 0.53 per cent nitrate is capable of reducing excess nitrate to nitrite.

Method: One series of samples was set up as in experiment 1 for maximum nitrite production. A second series was set up after adding 10 cc. distilled water containing 0.25 gr. sodium nitrate to each gram of hay, making a total of 2.15 per cent nitrate per gram of hay. A third series was set up after adding water containing 0.5 gr. sodium nitrate to each gram of hay, making a total of 3.77 per cent nitrate per gram of hay. Each of the three series was tested quantitatively for nitrite after twenty hours.

Results: As in experiment 1, the 0.53 per cent nitrate hay produced the equivalent of 0.22 per cent nitrite, calculated as sodium nitrite. The 2.15 per cent nitrate hay produced 0.95 per cent nitrite. The 3.77 per cent hay produced 1.33 per cent nitrite.

Conclusions: Oat hay will reduce excess nitrate to nitrite in the presence of distilled water and air. Hay containing 3.77 per cent nitrate would contain 18.1 Gm. nitrate per pound, and may convert nitrate to nitrite so that approximately 7 Gm. of nitrite per pound of hay would be present after twenty hours of moistening.

Bradley *et al.*[2] determined the approximate lethal dose of potassium nitrate for cattle to be 25 Gm. cwt. of animal. The therapeutic dose of nitrites is 1/10 to 1/20 of the dose of nitrate, and the lethal dosage probably falls within the same range. In the case of the turkey cited earlier, 50 gr. of KNO_3 was not fatal, but 7 gr. of nitrite was. If a dry feed such as oat hay, pigweed, or etc., should contain a percentage of nitrate slightly under that required to produce poisoning, it seems logical that such a feed might, twenty hours after being moistened by rain or snow, develop nitrite in quantities sufficient to make the feed highly toxic.

SUMMARY

Poisoning associated with recent rain has been reported in cattle feeding on oat hay, barley hay, wheat hay, and pigweed.

Several turkey flocks were found to be afflicted with nitrite poisoning following a rainstorm.

Evidence in botanical and agronomical literature shows that plants contain a substance which may reduce nitrate to nitrite.

Experiments conducted with oat hay moistened with distilled water show that the nitrate in dry oat hay and in turkey mash is reduced to nitrite, and that the hay is capable of reducing excess nitrate to nitrite, approximately ½ the nitrate being reduced to nitrite and present as such after twenty hours.

Poisoning associated with the ingestion of high nitrate hays following rain may be caused by the nitrite produced in the hay.

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Experimental Vaccination for Prevention of the Avian Leucosis Complex

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THE LITERATURE recording research in any phase of the avian leucosis complex reveals but few instances in which any attempt has been made to develop a vaccine for the prevention of this disease complex. Conclusions drawn from most work in this field are that at present no drugs, medicines, special feeds, tonics, vitamins, bacterins, or vaccines are known to have specific value in its cure or prevention. Perhaps the many points of similarity to tumors, and with but meager support for prophylactic treatment of these, even where well-established transmissible oncogenic agents are known to be the cause, has discouraged extensive experimentation in this direction. The recent investigations by Duran-Reynals and Estrada[1] indicate that an antibody-like factor present in the serum of adult chickens will modify or suppress lesions of the Rous sarcoma in experimentally infected chicks. It was noted that even in the early work of Rous and Murphy they observed that fowl in which tumors had regressed were resistant to subsequent inoculation, and that serums of these fowl neutralized the transmitting agent. Kabat and Furth,[2] several years later, found neutralizing antibodies against fowl-tumor agents in the serums of rabbits injected with heavy materials obtained by centrifugation of chicken tumors.

The work herein reported was started in 1938 when crystal violet was used to attenuate extracts of spleen tissue taken from birds affected with leucosis. The preparation of this vaccine was based upon the procedures described by McBryde and Cole[3] in connection with their work on hog cholera. The ability of the birds, vaccinated with this material, to withstand later artificial inoculations with leucotic materials was not significantly greater than that of unvaccinated controls. For the purpose of conserving space, data on these experiments are omitted.

In analyzing the results from these experiments several possible explanations for the failure of this vaccine might be pointed out, but in the light of work done on vaccination for the prevention of other virus diseases, particularly canine distemper, perhaps the failure to use a final dose of unattenuated virus is most important. An outstanding example of the opposing view, however, is the successful vaccination of horses against encephalomyelitis with greatly attenuated, if not dead, virus material. It was on the assumption that living virus is not necessary for the production of immunity to virus diseases that a series of birds were vaccinated, using materials from birds affected with various forms of leucosis in a manner similar to the Laidlaw-Dunkin method of vaccination for the prevention of canine distemper, *i.e.*, a final dose of living virus was sought to be used. Nine groups of birds were vaccinated, and an adequate number of similar birds left unvaccinated as controls. The vaccines were prepared and used as herein outlined, and the results are indicated in tables 2, 3, and 4.

Vaccine 1A was prepared from a composite extract of heart, liver, and spleen taken from a bird affected with leg paralysis (neurolymphomatosis). The extract was prepared by mincing the tissues and grinding them in a mortar with pestle and broken glass. Sufficient sterile physiological sodium chloride solution was added to make a liquid preparation. This was then strained through two layers of cheesecloth,

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and the pulp was discarded. The filtrate was then centrifuged for one-half hour in an angle centrifuge at approximately 3,000 r.p.m., and the supernatant liquid was passed through a Berkefeld N filter. The resulting filtrate was left at room temperature for twenty-four hours and then 1 cc. was injected into each bird intramuscularly.

Vaccine 1B was prepared from the spleen of a bird affected with leg paralysis (neurolymphomatosis). Five grams of spleen tissue with 45 cc. of sterile salt solution were ground in a mortar with pestle and broken glass as with vaccine 1A. This ground material was strained through three layers of cheesecloth. To the filtrate, which amounted to 35 cc., 0.2 cc. of formalin was added. After storing in the refrigerator for twenty-four hours, 1 cc. of this formalinized material per bird was inoculated intramuscularly.

Vaccine 1C was made from the spleen of a bird affected with leg paralysis (neurolymphomatosis) in a manner similar to 1B except that after straining through cheesecloth and centrifuging, the supernatant material was passed through a Berkefeld N filter and no formalin was added. The filtrate was kept in the refrigerator twenty-four hours while being tested for sterility, and then 0.1 cc. per bird was injected intradermally into the wattle.

The method of preparing the various vaccines used in these experiments was similar to the method of preparing vaccines 1A, 1B, and 1C, with the variations noted in table 1.

DISCUSSION OF PROCEDURE AND RESULTS

All of the birds were kept until they were approximately 1 year old and from eight to ten months after being vaccinated. At the end of the experiments, all birds were subjected to a careful autopsy. In any case where gross lesions and blood smears were insufficient for establishing a diagnosis, appropriate tissues were fixed and stained for microscopic examination. Vaccinated

TABLE I—Data on Preparation of Vaccines

VACCINE NUMBER	TISSUES USED	DILUENT	FILTRATION	REFRIGERATION	FORMALIN
2 A	Spleen, v.l.	NaCl*	None	10C. 24 hr.	0.4%.
2 B	Spleen, v.l.	NaCl	None	10C. 24 hr.	0.4%.
2 C	Spleen, heart, o.l.	NaCl	Berkefeld V	None	None.
3 A	Spleen, n.l. and o.l.	NaCl	None	10C. 24 hr.	0.4%.
3 B	Spleen, v.l. and m.l.	NaCl	None	10C. 24 hr.	0.4%.
3 C	Spleen, e.l.	NaCl	Berkefeld V	None	None.
1 X	Heart, kidney, spleen, lung, v.l.	50% NaCl-glycerine	None	10C. 72 hr.	0.4%.
2 X	Heart, liver, spleen, o.l. and v.l.	NaCl	None	10C. 72 hr.	0.4%.
3 X	Heart, spleen, lung, o.l.	50% NaCl-glycerine	Berkefeld V	Iced during preparation	None.
1 Y	Heart, liver, spleen, o.l. and v.l.	NaCl	None	10C. 20 days	0.4%.
2 Y	Spleen, o.l. and v.l.	NaCl	None	10C. 30 days	0.4%.
3 Y	Heart, spleen, lung, o.l.	50% NaCl-glycerine	Berkefeld V	10C. 21 days	None.
1 Z	Heart, liver, spleen, o.l. and v.l.	NaCl	None	10C. 6 weeks	0.4%.
2 Z	Heart, spleen, o.l.	buffered saline-glycerine	None	10C. 3 days	0.4%@37C. 1 hr.
3 Z	Lung, heart, spleen, o.l.	buffered saline-glycerine	None	10C. 11 days†	None.
1 L	Heart, lung, spleen, liver, v.l.	NaCl	None	10C. 4 days	0.4%.
2 L	Heart, lung, kidney, liver, spleen, v.l.	buffered saline-glycerine	Berkefeld N	Iced during preparation	None.
1 M	Heart, lung, liver, spleen, kidney, v.l.	NaCl	None	10C. 11 days	0.4%.
2 M	Spleen, kidney, v.l.	NaCl	None	10C. 18 days	0.4%.
3 M	Heart, lung, liver, spleen, v.l.	buffered saline-glycerine	Berkefeld N	5C. 18 days	None.
1 N	Heart, lung, liver, spleen, kidney, v.l.	NaCl	None	10C. 46 days	0.4%.
2 N	Spleen, kidney, v.l.	NaCl	None	10 C. 53 days	0.4%.
3 N	Heart, lung, liver, spleen, m.l.	buffered saline-glycerine	Berkefeld N	Iced during preparation	None.

v.l. = visceral lymphomatosis.
o.l. = ocular lymphomatosis.
n.l. = neurolymphomatosis.
m.l. = myeloid leucosis.
e.l. = erythroleucosis.
*0.85 per cent.
†No refrigeration for about forty-eight hours in this period.

and control birds in all of the experiments were fed and managed as identically as possible.

In group 1 (A, B, and C), 2 vaccinated birds developed the disease from pen contact. These birds were 4 weeks old when vaccinated and there is a possibility that they were infected before vaccination, although all the usual precautions were taken to prevent this.

Group 2 (A, B, and C) is interesting in that no vaccinated bird developed the disease. This vaccinal material came from cases with visceral and ocular lymphomatosis while that for group 1 came from cases with neurolymphomatosis. The exposure following was more severe in that some birds were inoculated with fresh material in addition to being exposed by pen contact to the disease.

TABLE 2—Vaccination Experiments A, B, and C Summarized

NUMBER OF BIRDS AND AGE AT TIME OF FIRST DOSE	DATE OF FIRST DOSE, AND VACCINE USED	DATE OF SECOND DOSE, AND VACCINE USED	DATE OF THIRD DOSE AND VACCINE USED	TIME AND TYPE OF EXPOSURE AFTER VACCINATION	RESULTS
17 4 weeks	2/14/42 1 A	2/24/42 1 B	3/11/42 1 C	Pen contact with two affected birds, v.l., 6/1/42 to 12/1/42	1 male died 7/9/42, v.l. 1 female died 8/31/42, v.l.
18 4 weeks	3/24/42 2 A	4/4/42 2 B	4/17/42 2 C	Pen contact, and some inoculated with v.l. material, 8/11/42	No form of the disease developed. 3 males and 5 females died with some form of leucosis.
46 4 weeks	4/16/42 3 A	4/28/42 3 B	5/19/42 3 C	Pen contact, and some inoculated with v.l. material, 8/11/42	1 male died 10/5/42, v.l. 1 female died 1/15/43, v.l.

12 control birds, left unvaccinated, were exposed by pen contact and inoculated 8/11/42, as were group 3.
v.l. = visceral lymphomatosis.

In the light of the later experiments, it appears that perhaps the failure of the vaccine used on group 3 (A, B, and C) to protect may have been because the formalinized vaccine was not held for a sufficient length of time before using to sufficiently attenuate or kill the virus, and that infection rather than immunity was provoked by the first dose.

The X series is also interesting in that no case of leucosis developed among the vaccinated birds. Since these vaccines were made from the same materials as the Y series, the better results obtained might have to do with the method of handling the materials that constituted the third dose. It will be noted that in the Y series this material was stored in a refrigerator at 10 C. for twenty-one days, whereas, in the X series it was used immediately after its preparation. The failure of the Z series was striking. Due to some difficulty with the electric current, the refrigerator was without current for approximately forty-eight hours May 15 and 16. The apparent failure of this vaccine as shown in table 3 might be attributed to partially killed virus due to lack of proper refrigeration before injection of last dose.

An attempt has been made to see if any degree of immunity against the various forms of the avian leucosis complex could be produced by using materials from various forms in various ways. Unfortunately, this could neither be carried out completely nor extensively because the proper materials were not always available at the proper time. In spite of these limitations, this preliminary work shows some promise, especially in the groups where no vaccinated birds developed the disease. In the N group, both vaccinated and control birds were challenged with the Beltsville strain-A virus, which was very potent for young chicks, but appeared to be less virulent for older birds. In this group, particularly, there was a marked difference in favor of the vaccinated birds to withstand artificial inoculations.

It is at once evident that if a vaccine for

this purpose shall have any significant, practical use, it must in some way be standardized as to content, and it must be in some way preserved to retain its potency for future use. In an attempt to solve this phase, the Beltsville strain-A virus was tried on chicken embryos and baby chicks, and the vaccines thus produced subjected to desiccation at low temperature in vacuum.

The intravenous injection of embryos, according to the method described by Eichhorn,[4] was not tried extensively because of the difficulty in making satisfactory injections and because of the difficulty in reducing the embryos to a suitable powder. Baby chicks were found to be more suitable for this purpose. They can be injected in the wing vein quite successfully when only a few days old. The progress of the disease can be determined by examining a stained blood smear made every few days. With the Beltsville strain-A virus it usually takes from two to four weeks to establish the disease. When the blood contains a preponderance of immature lymphoid cells (hemocytoblasts) the chick may be bled by cardiac puncture to obtain practically all of its blood. This was done and the blood placed in sterile Petri dishes and frozen, as soon as clotting had taken place, in dry ice (CO_2). The Petri dishes containing the blood were placed in a desiccator containing phosphorus pentoxide (P_2O_5) and the temperature reduced gradually to about —20 C. in about an hour. At approximately that stage, the vacuum pump was started and vacuum of about 29 inches of mercury on the gauge was produced. In about three hours, the temperature was reduced to —50 C., or below. In from twenty to twenty-four hours the blood was completely desiccated and was then ground in a sterile mortar to a fine powder. This powder was then placed in sterile glass tubes that had previously been constricted to facilitate sealing. After filling the tubes about half way to the constricted area with the powdered material, they were attached to the pump and when a vacuum of about 29 inches of mercury on the gauge had been reached, the constricted portion of the tube was sealed with a Bunsen burner. The seal could be tested for flaws or leaks with a dissecting needle. After sealing the desiccated material under vacuum, it was stored

TABLE 3—Vaccination Experiments X, Y, and Z Summarized

No. of birds and age at time of first dose	Date of first dose, and vaccine used	Date of second dose, and vaccine used	Date of third dose, and vaccine used	Time and type of exposure after vaccination	Results
30 2 weeks	3/15/43 1 X	3/25/43 2 X	4/8/43 3 X	Injected n.l., 5/31/43 into 15 Pen contact with v.l., 10/1/43	No form of the disease developed.
30 2 weeks	4/9/43 1 Y	4/19/43 2 Y	4/29/43 3 Y	Injected n.l., 6/2/43 into 14 Pen contact with v.l., 11/15/43	2 died of v.l.
30 2 weeks	5/3/43 1 Z	5/10/43 2 Z	5/18/43 3 Z	Injected v.l., 6/12/43 into 13 Pen contact with v.l., 9/30/43.	8 died of some form of the disease.
Controls for X series 13				6 injected as above and all by pen contact as in Group X.	3 injected birds and 1 other developed leucosis.
Controls for Y series 26				8 injected as above and all by pen contact as in group Y.	3 injected birds and 2 others developed leucosis.
Controls for Z series 24				10 injected as above and all by pen contact as in group Z.	4 injected birds developed leucosis.

v.l. = visceral lymphomatosis.
n.l. = neurolymphomatosis.

at —10 C. until used. Such desiccated material has remained potent for a period of three months.

Liver and spleen also can be used, but it is more difficult to grind these sufficiently to reduce the connective tissue present to a suitable powder. Such materials are less suitable for intravenous or intradermic injections, but for vaccines to be used subcutaneously they are satisfactory.

Vaccination with Frozen Materials Desiccated in Vacuum.—Twenty-six week-old chicks were vaccinated with desiccated materials obtained from chicks injected with the Beltsville strain-A virus.

The first dose consisted of powdered spleen and liver suspended in sterile physiological salt solution, using one gram of the powdered material to 5 cc. of salt solution. Sufficient formalin was added to make 0.4 per cent and this formalinized material was then kept in the refrigerator for ten days, and shaken several times during this period. It was then used in 0.5 cc. amounts per chick subcutaneously in the region of the breast. Fourteen days later, desiccated blood from the same source was reconstituted by the addition of sterile distilled water and injected intradermally in the wattle in 0.1 cc. amounts per chick. Six weeks following the last injection, 15 of these vaccinated chicks and 10 similar control chicks were each inoculated intravenously with 0.5 cc. of reconstituted powdered blood. These injections were made Dec. 18, 1944. Four, five, and six weeks, respectively, following these injections 3 of the control birds developed the disease. Up to that time none of the vaccinated birds had been affected.

This work should be expanded, and it is hoped that this can be done within the next few months, realizing that this work is not sufficiently advanced to allow definite conclusions. In all cases, the vaccinated and control birds should be challenged earlier

TABLE 4—Vaccination Experiments L, M and N Summarized

No. of birds and age at time of first dose	Date of first dose and vaccine used	Date of second dose and vaccine used	Date of third dose and vaccine used	Time and type of exposure after vaccination	Results
27 2 weeks	3/15/44 1-L	3/29/44 2-L	None used	Injected 23 with myeloid blood and v.l., 6/1/44, pen contact with v.l., 6/8/44.	1 bird developed a tumor on skin of neck 5/15/44. This is not considered as a part of the leucosis-complex.
28 2 weeks	4/7/44 1-M	4/14/44 2-M	4/24/44 3-M	Injected 16 with myeloid material, 6/1/44, and 5 with blood from strain A leucosis, 8/30/44. All by pen contact, 9/19/44.	1 bird developed v.l., 11/6/44.
30 2 weeks	5/18/44 1-N	5/25/44 2-N	6/1/44 3-N	Injected 18 with v.l., 7/21/44; 6, with blood from strain A leucosis, 8/30/44. All by pen contact, 9/19/44.	1 of the vaccinated birds developed leucosis.
Controls for L series 22				Injected 18 as above, and all by pen contact, as in series L.	Only 1 bird developed o.l., 1/8/45.
Controls for M series 20				Injected 11 and 3 as above, and all by pen contact, as in series M.	1 developed v.l., 7/21/44, and 1 hemocytoblastic leucosis, 9/29/44.
Controls for N series 22				Injected 10 and 8 as above, and all by pen contact, as in series N.	6 died of various forms of leucosis.

v.l. = visceral lymphomatosis.
o.l. = ocular lymphomatosis.

after vaccination with material that appears to bring about the explosive outbreaks, frequently noted under field conditions. This should also be done by using fresh material of the Beltsville strain-A virus. This report seems justified, however, in that other workers may wish to undertake work along these lines. It would seem especially desirable to try out some of these vaccination procedures in the field where the disease occurs in a severe form. Repetition and expansion under laboratory conditions are likewise necessary before final conclusions can be drawn.

TABLE 5—Summary of Vaccination Results

VACCINES USED	TYPES OF MATERIAL	RESULTS
1 A—1st dose	n.l.	2 of 17 vaccinated birds developed v.l.
1 B—2nd dose	n.l.	2 of 12 control birds developed v.l.
1 C—3rd dose	n.l.	
2 A—1st dose	v.l.	None of 18 vaccinated birds developed any form of leucosis.
2 B—2nd dose	v.l.	2 of 12 control birds developed v.l.
2 C—3rd dose	o.l.	
3 A—1st dose	n.l. and o.l.	8 of 46 vaccinated birds developed some form of leucosis.
3 B—2nd dose	v.l. and m.l.	2 of 12 control birds developed v.l.
3 C—3rd dose	e.l.	
1 X—1st dose	v.l.	None of 30 vaccinated birds developed any form of leucosis.
2 X—2nd dose	o.l. and v.l.	4 of 13 controls developed some form of leucosis.
3 X—3rd dose	o.l. and v.l.	
1 Y—1st dose	o.l. and v.l.	2 of 30 vaccinated birds developed leucosis.
2 Y—2nd dose	o.l. and v.l.	3 of 26 control birds developed leucosis.
3 Y—3rd dose	o.l.	
1 Z—1st dose	o.l. and v.l.	3 of 30 vaccinated birds developed leucosis.
2 Z—2nd dose	o.l.	4 of 24 control birds developed leucosis.
3 Z—3rd dose	o.l.	
1 L—1st dose	v.l.	1 of 27 vaccinated birds developed a tumor on neck (fibrosarcoma).
2 L—2nd dose	v.l.	1 of 22 control birds developed o.l.
1 M—1st dose	v.l.	1 of 28 vaccinated birds developed v.l.
2 M—2nd dose	v.l.	2 of 20 control birds developed leucosis.
3 M—3rd dose	v.l.	
1 N—1st dose	v.l.	1 of 30 vaccinated birds developed v.l.
2 N—2nd dose	v.l.	6 of 22 control birds developed some form of leucosis.
3 N—3rd dose	m.l.	

v.l. = visceral lymphomatosis.
o.l. = ocular lymphomatosis.
n.l. = neurolymphomatosis.
m.l. = myeloid leucosis.
e.l. = erythroleucosis.

SUMMARY AND CONCLUSIONS

Data are presented on the preparation and use of several vaccines to determine their value in the prevention of the so-called avian leucosis complex. A preliminary and inadequate experiment with crystal-violet attenuated material gave results that were not sufficiently encouraging to warrant expansion of the work. The experiments in which formalinized tissues were used for the first injections, followed by a final injection of unattenuated material intradermally, involved the use of 431 birds, of which 282 were vaccinated and 149 were unvaccinated and served as controls. Tables 1, 2, 3, and 4 give the essential data on the materials used in the various vaccines, the age of the birds at time of vaccination, and the time and type of exposure used to determine their effectiveness. The results are summarized in table 5. Vaccines in series 2A, 2B, 2C, and series 1X, 2X, 3X, are of significance, as no vaccinated birds in these series developed any form of the disease. Vaccines in series 1N, 2N, 3N are perhaps even more significant in that the type of exposure was more severe, and more controls developed the disease. Finally, the limited work done with the use of frozen, desiccated Beltsville strain-A virus is encouraging and should be expanded.

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Studies on Certain Filtrable Viruses
VIII. Viability of Chicken Embryo Fowl Pox Virus

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FOLLOWING the propagation of fowl-pox virus on the chorioallantoic membrane of developing chicken embryos by Woodruff and Goodpasture,[1] the possible advantages of chorioallantoic membrane virus over skin lesion virus for immunizing chickens against fowl-pox was reported by Brandly,[2] Dunlap,[3] and Brandly and Dunlap.[4] Subsequently, the virus obtained from the entire chicken embryo was demonstrated by Thorning, Graham, and Levine[5] to yield ten times more virus for mass immunization than chorioallantoic membrane alone. It appears that three forms of fowl-pox, virus, *i.e.*, skin lesion, chorioallantoic membrane, and entire embryo may be employed successfully in the field.

These three forms of virus are stored in the desiccated state. The viruses are triturated in the laboratory before packaging and immediately prior to field use suspended in diluents such as distilled water or physiological saline solution. Apparently, little attention has been given to the preparation and preservation of fowl-pox virus suspended in diluents that could be packaged ready for application in the field. The purpose of this paper is to record observations on the duration of viability and the immunogenic properties of chicken embryo fowl-pox virus suspended and stored in different diluents.

EXPERIMENTAL FOWL-POX VIRUS SUSPENSIONS

A single strain of fowl-pox virus was employed. Propagation was by the method described by Thorning, Graham, and Levine.[5] Inoculated chorioallantoic membranes of fertile eggs showing typical fowl-pox lesions were harvested for chorioallantoic virus. The entire embryo was utilized, including only embryos showing gross lesions on the chorioallantois. The chorioallantois and entire embryo viruses were then desiccated over calcium chloride, ground separately in sterile mortars, and diluted 1 to 100, weight per volume, in different diluents, and stored at 5 C. The diluents employed included heavy, white mineral oil (U.S.P.), mineral oil (U.S.P.) buffered, mineral oil (U.S.P.) plus 0.1 per cent chloroform, soybean oil, buffered soybean oil, glycerin (C.P.) 50 per cent, mineral oil (U.S.P.) plus 0.1 per cent formaldehyde, mineral oil (U.S.P.) plus 0.1 per cent phenol, mineral oil (U.S.P.) plus sodium azide 1 to 15,000 and brilliant green 1 to 50,000, thioglycollate broth plus 0.25 per cent phenol, and glycerin (C.P.).

At intervals, each virus suspension was inoculated by the three-stick method in the wing patagium of 4 chickens susceptible to fowl-pox. Observations for take reactions were made at the end of the first, second, and third weeks following inoculation. At the end of the third week, the chickens were exposed to fowl-pox virus by the feather follicle method to determine if immunity had been produced. Some of the virus preparations were repeatedly tested until no evidence of protection was observed.

The chorioallantoic fowl-pox virus, in mineral oil and buffered mineral oil, proved viable for thirteen months. Further tests were not made on these suspensions. Chorioallantoic virus in mineral oil plus 0.1 per cent chloroform proved viable for five months. Entire embryo fowl-pox virus in mineral oil and buffered mineral oil proved viable for five months, while entire embryo fowl-pox virus in mineral oil plus 0.1 per cent chloroform proved viable for three months. Chorioallantoic fowl-pox

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virus in soybean oil and buffered soybean oil proved fully viable for six and four months, respectively, while chorioallantoic virus in 50 per cent glycerin proved viable for five months. Entire embryo fowl-pox virus in 50 per cent glycerin proved viable for three months. Chorioallantoic fowl-pox virus in mineral oil plus 0.1 per cent formaldehyde, mineral oil plus 0.1 per cent phenol, mineral oil plus sodium azide with brilliant green, glycerin, and thioglycollate broth plus 0.25 per cent phenol rendered the virus inert. Of the various suspensions employed, fowl-pox virus in mineral oil and mineral oil with buffer appeared most satisfactory from the standpoint of duration of viability (*see* table 1).

IMMUNIZATION OF FARM FLOCKS

To determine the immunizing ability of the chorioallantoic and entire embryo fowl-pox viruses suspended in mineral oil, 3 separate lots of fowl-pox virus suspended in mineral oil were employed to inoculate 7 apparently healthy farm flocks including 15,609 chickens. The fowl ranged in age from 9 to 16 weeks. Flocks 1, 2, 3, and 4, composed of 2,684 White Leghorn chickens, were inoculated by the three-stick method in the wing patagium with chorioallantoic virus in mineral oil. The experimental vaccine was stored at 5 C. for five to nine weeks before being used. In the fifth and sixth flocks, comprising 6,935 chickens, mainly White Leghorns, approximately one-half of the birds in each flock were inoculated with a portion of the vaccine used to immunize flocks 1, 2, 3, and 4. The

TABLE 1—Viability of Chicken-Embryo Fowl-Pox Virus Suspended, 1 to 100, in Different Diluents*

FOWL-POX VIRUS	MONTHS												
	T.P.	1	2	3	4	5	6	7	8	9	10	11	12	13
CA in mineral oil	++	++	++	++	++	++	++	++	++	++	++	++	++	++
CA in buffered mineral oil†	++	++	++	++	++	++	++	++	++	++	++	++	++	++
CA in mineral oil + 0.1% chloroform	++	++	++	++	++	++	0							
EE in mineral oil	++	++	++	++	++	++	0							
EE in buffered mineral oil†	++	++	++	++	++	++	+	0						
EE in mineral oil + 0.1% chloroform	++	++	++	++	0									
CA in soybean oil	++	++	++	++	++	++	++	0						
CA in buffered soybean oil†	++	++	++	++	++	+	0							
CA in 50% glycerin	++	++	++	++	++	++	0							
EE in 50% glycerin	++	++	++	++	0									
CA in mineral oil + 0.1% formaldehyde	+	0												
CA in mineral oil + 0.1% phenol	+	0												
CA in mineral oil + sodium azide, brilliant green‡	+	0												
EE in mineral oil + 0.1% phenol	+	0												
EE in mineral oil + sodium azide, brilliant green‡	+	0												
CA in thioglycollate broth + 0.25% phenol	+	0												
EE in mineral oil + 0.1% formaldehyde	0													
CA in 100% glycerin	0													
EE in 100% glycerin	0													

CA = chorioallantoic virus.
EE = entire embryo virus.
++ = 4 chickens showing reaction.
* = all preparations stored at 5 C.
† = Sorensen's buffer salts to approximate a pH of 7.0.
‡ = sodium azide 1-15,000; brilliant green 1-50,000.
+ = 1 to 4 chickens showing reactions.
0 = no chickens showing reaction.
T.P. = time of preparation.

remaining birds in flocks 5 and 6 were inoculated with chorioallantoic virus suspended in mineral oil and stored at 0 C. for twelve to fifteen weeks. The three-stick method in the wing patagium was employed. In flock 7, comprising 5,990 White Leghorns and New Hampshire Reds, entire embryo virus in mineral oil stored twelve to thirty-six hours at 5 C. was employed by the three-stick method in the wing patagium or the lateral aspect of the thigh. For appraisal of the immunity produced, between the seventh and eleventh days after inoculation, a representative number of the birds from each of the 7 flocks was examined for take reactions. Also, a number of birds from flocks 6 and 7 was obtained for artificial exposure to virus.

In flocks 1, 2, 3, and 4, inoculated with chorioallantoic virus in mineral oil stored five to nine weeks at 5 C., takes ranged from 78.5 per cent to 100 per cent, with an average of 90.45 per cent in 2,684 birds. In flocks 5 and 6, inoculated with chorioallantoic virus stored at 5 C. in mineral oil and the chorioallantoic virus in mineral oil stored twelve to fifteen weeks at 0 C., takes ranged from 91.65 per cent to 97.88 per cent with an average of 95.19 per cent in 6,935 birds. In flock 7 including 5,990 birds inoculated with entire embryo virus suspended twelve to thirty-six hours at 5 C. in mineral oil, observations for takes showed 96.61 per cent reactions. In a total of 15,609 birds, approximately 10 per cent were examined following vaccination and 93.4 per cent showed take reactions.

Ninety-six per cent of 300 birds from flock 6, artificially exposed seven to nine weeks following inoculation, proved immune. Approximately the same percentage, 96.27, of 118 artifically exposed birds from flock 7 proved immune three weeks following inoculation (*see* table 2).

TABLE 2—Results of Inoculation of Flocks of Chickens with Fowl-Pox Virus Suspended, 1 to 100, in Mineral Oil

VACCINE	FLOCK NO.	NO.	NO. EXAMINED	TAKES (%)	NO. EXPOSED*	PROTECTED (%)
CA, 5 to 9 weeks at 5 C.	1	420	120	100		
	2	629	127	88.11		
	3	329	90	78.50		
	4	1,306	103	92.23		
	1 to 4	2,684	440	90.45		
CA, 6 to 9 weeks at 5 C.	5	2,935	520	97.88		
CA, 12 to 15 weeks at 0 C.	6	4,000	395	91.65	300	96.00
	5 and 6	6,935	915	95.19		
EE, 12 to 36 hours at 5 C.	7	5,990	207	96.61	118	96.27
	1 to 7	15,609	1,562	93.40		

CA = chorioallantoic virus in mineral oil.
EE = entire embyro virus in mineral oil.
* = entire embyro virus by three-stick method.

SUMMARY

The duration of viability of chicken-embryo fowl-pox virus, suspended in different diluents, and results of field experiments on production of immunity by inoculation of farm flocks with chicken-embryo fowl-pox virus, suspended in mineral oil, are reported. Duration of viability of chorioallantoic virus varied from time of preparation when suspended in pure glycerin or thioglycollate broth with phenol to at least thirteen months when suspended in mineral oil or buffered mineral oil. Entire embryo virus showed viability of from five to six months in mineral oil or buffered mineral oil. Inoculation of 15,609 chickens with chicken-embryo fowl-pox virus, including chorioallantoic virus and entire embryo virus suspended in mineral oil, resulted in 93.40 per cent takes. When 418 birds from 2 of the 7 flocks were selected for artificial exposure, 96.14 per cent resisted.

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Sulfaquinoxaline in Preventing Upper Respiratory Infection of Chickens Inoculated with Infective Field Material Containing Pasteurella Avicida

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STUDIES in which sufathiazole and some other sulfonamides have been used[1,2] have failed to show that any give promise of value in the prevention or control of endemic Pasteurella infection (endemic fowl cholera) of the respiratory tract of chickens. Infectious coryza (*Hemophilus gallinarum* infection), which is frequently mistaken for enzoötic fowl cholera, differs in that infected birds respond to sulfathiazole medication. Sulfamerazine (unpublished data) has shown some value in preventing infection in birds under experimental conditions, but is too toxic for prolonged, continuous use in chickens when given in effective amounts.

Recently, through the courtesy of Merck and Company, one of the newer sulfonamides, sulfaquinoxaline, was called to our attention as having possible use in poultry.

The first study was undertaken to determine whether sulfaquinoxaline would hasten the recovery of 5 maturing White Leghorn pullets showing a simple nasal discharge as a result of enzoötic Pasteurella infection. Each bird was given 250 mg. of sulfaquinoxaline daily for nine days without any apparent beneficial results or any visible toxicity. It was apparent from the study that sulfaquinoxaline did not have any appreciable curative value. Since sulfaquinoxaline did not appear to have toxic properties in the proportions used, it was planned to study it further to determine whether or not it would prevent infection under experimental conditions.

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MATERIALS AND METHODS

Six different experimental groups consisting of Rhode Island Red cockerels and pullets, approximately 3 months old, were used in these studies.

The field material for infecting groups 1 and 2, was obtained from the ear of a naturally infected bird which had been under observation over a considerable period of time and from which *Pasteurella avicida* had been isolated.

The material used for groups 3, 4, 5 and 6, was obtained from a farm where enzoöitic Pasteurella infection had recurred for several years, and constituted a source of material which had been used for other studies of a similar nature. *P. Avicida* had repeatedly been isolated from affected birds from this flock.

All chickens were inoculated by vigorously swabbing their nasal passages, using wooden applicators which had been dipped in the exudate from infected birds. Such procedure rep-

Table 1—Results of Inoculating Unmedicated Birds and Birds Receiving 0.1 and 0.05 Per Cent Sulfaquinoxaline in Their Feed, Using Field Infective Material Containing Pasteurella Avicida

Experimental group	Unmedicated birds		Medicated birds			
			0.1% sulfaquinoxaline in feed		0.05% sulfaquinoxaline in feed	
	No. inoculated	No. infected	No. inoculated	No. infected	No. inoculated	No. infected
1 (cockerels)	5	5*	10	2†	0	0
2 (cockerels)	5	3* 1‡	10	2†	0	0
3 (cockerels)	10	8*	17	0	0	0
4 (pullets)	5	4* 1‡	10	0	10	0
5 (pullets)	5	5*	10	0	10	1‡
6 (pullets)	5	5*	8	1‡	8	0
Total	35	32	65	5	28	1
Percentage		91+		7.6		3.5

*Severe. Birds had distended nasal passages, swollen faces and/or swollen wattles. P. avicida repeatedly isolated from such birds.
†Mild. Slight nasal discharge only symptom shown.
‡Definite nasal discharge without any swellings of face or wattle.

resents an exposure much more severe than that occurring from natural contact.

The control groups received unmedicated mash, whereas the medicated birds received mash containing 0.1 or 0.05 per cent sulfaquinoxaline* as indicated. All birds were examined individually each day for evidence of infection.

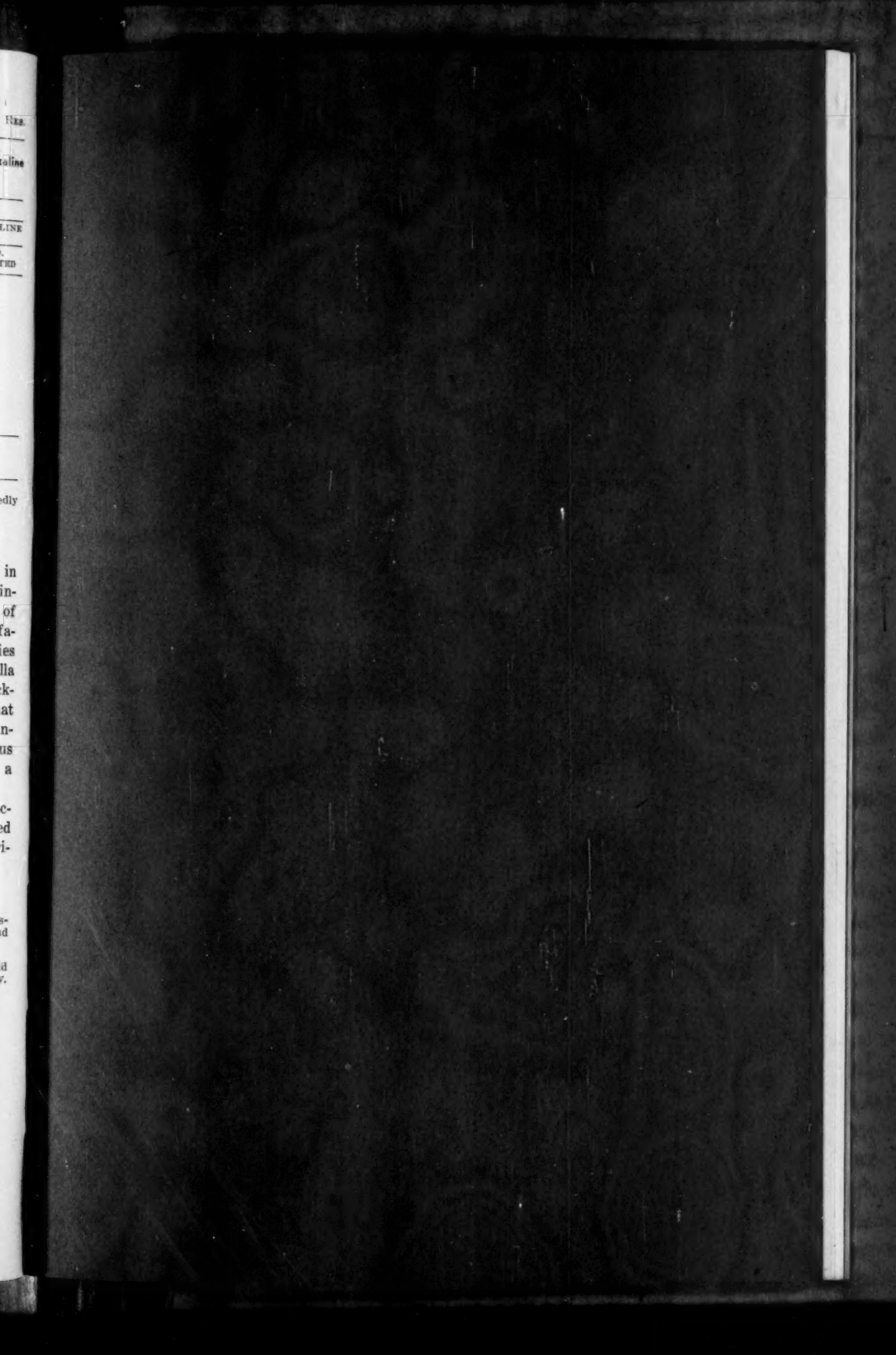
Discussion and Summary

The results obtained in 6 different experimental groups of chicks are shown in table 1. Thirty-five birds served as inoculated untreated controls and 93 were given medicated feed before and after being inoculated. Thirty-two of the 35 inoculated controls (91%) developed the infection. Most of them showed severe manifestations of the disease. Six of 93 birds receiving 0.1 and 0.05 per cent sulfaquinoxaline in their feed developed mild symptoms of infection characterized by mere traces of nasal discharge. From these studies sulfaquinoxaline would seem to offer possibilities in the prevention of enzoötic Pasteurella infections of the respiratory tract of chickens, providing further studies show that when given at the levels cited it is not injurious to the bird, or is less injurious than the disease would be if all birds of a flock became affected.

Sulfaquinoxaline does not have any recognizable curative properties as indicated by the results of the preliminary experiment.

*According to the Merck Institute for Therapeutic Research, the average blood concentration of chickens fed mash containing 0.1 per cent sulfaquinoxaline was 9 mg. per cent. Feed with 0.05 per cent of the drug averaged 4 mg. per cent.

References

[1]Delaplane, J. P.: The Differentiation of the Respiratory Diseases of Chickens. Rhode Island Agric. Exper. Sta. Bull. 288, (1943): 1-33.

[2]Wernicoff, N. E., and Goldhaft, T. M.: The Field Use of Sulfathiazole in Some Diseases of Poultry. Cornell Vet., 34, (1944): 199-213.